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The evolution of salamander mimicry: Predators, prey, and perception

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The evolution of salamander mimicry: Predators, prey, and perception

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ABSTRACT

Batesian mimicry is an interspecific relationship in which a palatable species, the mimic, avoids attacks from predators by resembling an unpalatable species, the model. Mimicry has long been studied to understand the evolutionary dynamics of adaptation, yet many factors affecting the co-evolution of mimics and models are understudied in natural systems. In the first portion of this dissertation, I describe mimicry between two salamander species in which the erythristic color morph of *Plethodon cinereus* (the mimic) resembles the juvenile eft stage of *Notophthalmus viridescens* (the model). I found that the coloration of mimics resembles that of models, particularly from the perspective of avian predators. I also discovered that while mimetic phenotype appears to have converged on model phenotype, selection from predators may drive models to appear distinct from mimics, particularly by appearing more conspicuous against common backgrounds.

In the second portion of this dissertation, I evaluate how variation in model toxicity may influence the evolution of mimicry by measuring the concentration of the neurotoxin, tetrodotoxin, in *N. viridescens* individuals from localities with varying degrees of mimicry. I found that despite significant variation in toxicity among localities, model toxicity did not predict either the occurrence of mimicry or variation in mimetic phenotype. These findings suggest that even though theoretical and experimental studies have predicted a link between model toxicity and mimicry, this relationship may not always be strong enough to influence mimic evolution in natural systems.

In the final portion of this dissertation, I identify selection on *P. cinereus* coloration by mammalian predators. I evaluated mammalian selection on *P. cinereus* color morphs by

measuring mammal attacks on clay replicas of *P. cinereus* and comparing these attacks against predictions of predator behavior under different hypotheses. Intriguingly, selection by mammalian predators was inconsistent with predictions from the hypothesis of mimicry. Instead, after developing a likelihood-based method for combining non-exclusive hypotheses of predator behavior, I found that mammals appear to use visual cues from salamander prey to preferentially attack familiar and conspicuous individuals. These results are important for the evolution of coloration in *P. cinereus* because they show that multiple predator species may differentially influence the evolution of coloration and thus complicate predictions of mimicry evolution.

CHAPTER 1

GENERAL INTRODUCTION

Overview

How do predators sense potential prey, and what are the implications of this perception for the evolution of prey phenotype? These questions have long captured the attention of evolutionary biologists, perhaps most clearly through the phenomenon of Batesian mimicry (Ruxton *et al*., 2004). In Batesian mimicry a palatable 'mimic' species closely resembles an unpalatable 'model' species in warning signal design (Bates, 1862). This form of mimicry was first observed in the mid-1800's when Henry Walter Bates recognized a striking resemblance in color and pattern between toxic and non-toxic Neotropical butterflies, despite their unique evolutionary histories (Bates, 1862). The evolution of Batesian mimicry is intimately tied to the antagonistic relationships between predators and their prey. Thus, through the study of Batesian mimicry we can better understand the evolutionary impact of predation on prey species, the evolution of convergent antipredator strategies, and the establishment of phenotypic diversity.

Much attention has been given to the conditions that lead to the initial establishment of Batesian mimicry (e.g. Fisher, 1930; Charlesworth & Charlesworth, 1975; Turner, 1988; Huheey, 1988), especially through tools that involve computer-generated models of predators and prey (e.g. Holen & Johnstone, 2006; Franks *et al.* 2009; Speed & Ruxton, 2010) or captive predators and artificial prey (e.g. Alatalo & Mappes, 1996; Lindstrom *et al.,* 1997; Lindstrom *et al.,* 2004). However, the factors that contribute to the maintenance of mimicry after its establishment are less well understood (Ruxton *et al.*, 2004). Research over the last

fifteen years has strived to fill this void, and has generated numerous hypotheses regarding the maintenance of mimicry (Kikuchi and Pfennig, 2013). This work has been dedicated to characterizing the evolution of mimicry in natural systems, and from this we have learned how mimicry evolves in a varied landscape of model and predator populations for numerous taxa, including coral snakes (e.g. Harper & Pfennig, 2007; Kikuchi & Pfennig, 2010), hoverflies (Penney *et al.* 2012), poison frogs (Darst *et al.* 2006), butterflies (Prudic & Oliver 2008), and other taxa (Ruxton *et al.*, 2004). Mimicry is a strategy some prey species use to avoid predation, and critically involves predator detection and recognition of prey as unpalatable. However in most cases of mimicry, predator perception of potential prey has not been investigated (but see Stoddard & Stevens, 2011). Additionally, many studies of mimicry consider only single factors such as model unpalatability, predator perception, and the influence of multiple predators. Thus, the combined impact of several factors is seldom investigated within single mimicry systems (but see Kikuchi & Pfennig, 2010a,b).

To understand which factors have been most influential in the evolution of a mimicry system, predator perception of model and mimic warning signals must first be characterized. Second, the evolution of mimicry must be evaluated as a consequence of evolving model and mimic warning signals and model unpalatability. Finally, mimicry must be considered as a single anti-predator defense that may not be directed at all potential predators, which allows for the possibility of novel selection from predators not consistent with mimicry. In this dissertation, I examine these factors in a putative Batesian mimicry system to understand how mimicry, anti-predator defense, and unpalatability evolve in salamanders.

The Hypothesis of Batesian Mimicry

The hypothesis of Batesian mimicry states that predators will avoid prey species that carry secondary defenses (Speed & Ruxton, 2005), and this protection from predation will extend to 'mimic' prey species that resemble defended prey, even if mimics themselves do not carry secondary defenses (Bates, 1862). Batesian mimicry is widespread and includes examples in hoverflies (models: hornets, wasps, and bees; Penney *et al.*, 2012), colubrid snakes (models: coral snakes; Greene & McDiarmid, 1981), dismorphian butterflies (models: Ithomiini butterflies; Bates, 1862), various fish (Cheney & Marshall, 2009), non-thorned plants (models: thorned plants; Lev-Yadun, 2003), and many other taxa (Ruxton *et al.,* 2004).

Theoretical and empirical work on predators and their prey have led to many predictions regarding mimicry (e.g., reviewed in Mallet & Joron, 1999; Speed, 1999; Ruxton *et al*., 2004; Pfennig & Mullen, 2010; Stoddard, 2012). With respect to warning signal design, three predictions are expected in most systems of Batesian mimicry. First, mimics are predicted to generally match model species in warning signal phenotype (Ruxton *et al.,* 2004). In particular, because predators are driving the evolution of mimicry, Batesian mimics are predicted to resemble their models in ways that potential predators will perceive (e.g. Kikuchi & Pfennig, 2010). Second, unpalatable models are often conspicuous and therefore aposematic (Roper & Redston, 1987; Ruxton *et al.*, 2004; Mappes *et al.,* 2005; but see Wüster *et al.,* 2004). When this is the case, mimics are predicted to evolve conspicuousness as well (reviewed in Ruxton *et al.,* 2004 and discussion in Franks *et al.,* 2009). Finally, since conspicuousness is perceived as the degree of contrast between a target and its background (Ruxton *et al.,* 2004), different backgrounds may influence prey detectability (e.g. Siddiqi *et*

al., 2004, Macedonia *et al*., 2009, Willink *et al.,* 2013). Therefore, the patterns of conspicuousness predicted between models and mimics may be background-specific.

Conspicuousness and Mimicry

Because conspicuous species stand out against their background they can incur costs that include increased attention from predators in addition to the cost of resources invested in conspicuousness. Taking these costs into account, it is unclear why conspicuousness should evolve at all (Ruxton *et al*., 2004). One explanation is that despite these costs, conspicuous coloration serves as an effective signal of unpalatability to potential predators, thereby reducing the likelihood that a potential prey will be recognized as a desirable meal (this relationship is termed 'aposematism'; e.g. Maan & Cummings, 2012). Conspicuousness is hypothesized to evolve in aposematic species to quickly train potential predators (Gittleman & Harvey, 1980; Roper & Redston, 1987; Lindström *et al*., 2001) and act as a mnemonic device for unpalatability (Guilford, 1986; Speed, 2000; Prudic *et al*., 2007). Conspicuousness may also ensure that aposematic individuals are not mistaken for palatable species (Fisher, 1930).

If conspicuousness evolves to distinguish unpalatable species from palatable species, mimicry, which eliminates the distinction between unpalatable and palatable species, may influence the evolution of conspicuousness. Here, the mimetic phenotype is selected to converge on model phenotype, while the model phenotype is selected to diverge from mimic phenotype (Franks & Noble, 2004). The resulting 'chase-away selection' may drive models to evolve new phenotypes (e.g. Poulton, 1890; Edmunds, 1974; Sherratt & Franks, 2005; Franks *et al*., 2009). Additionally, conspicuousness may evolve because conspicuous

warning signals are more costly for palatable mimics than unpalatable models, as a result of heightened attention from predators (Speed & Ruxton, 2010). Thus, conspicuousness in aposematic species may evolve to prevent palatable species from evolving perfect mimicry.

Model Unpalatability

In many Batesian mimicry systems, unpalatability is the consequence of toxic chemicals produced by the model (Ruxton *et al*., 2004). The toxicity of models may influence both the occurrence of mimicry (Endler, 1991; Lindström *et al*., 1997) and the evolution of mimetic phenotype (Duncan & Sheppard 1965; Goodale & Sneddon, 1977; Lindström *et al*., 1997). Mimicry is hypothesized to evolve and be maintained where models are most toxic (Endler, 1991; Lindström *et al*., 1997). Once established in a community, model toxicity may then influence the evolution of mimic phenotype in one of two ways. First, the classic expectation under natural selection is that in the presence of highly toxic models, mimics will evolve toward an ever more perfect resemblance of model phenotype (Fisher, 1930; Nur, 1970). In contrast to this expectation, mimics are frequently observed that closely, but not perfectly, resemble their models (imperfect mimicry *sensu*: Ruxton *et al.*, 2004). In such cases, model toxicity is thought to play a role by deterring predators from attacking individuals carrying the model phenotype, including mimics. Under the relaxed selection hypothesis, predators will increasingly avoid mimics as the penalty for mistakenly attacking models increases, particularly through increased toxicity (Schmidt, 1958; Duncan & Sheppard, 1965; Sherratt, 2002; Penney *et al*., 2012; Kikuchi & Pfennig, 2013). Thus, selection against imperfect mimics will decrease as models become more toxic, through a process termed ‘stimulus generalization’ (Duncan & Sheppard, 1965; Darst *et al*., 2006).

Support for the relaxed selection hypothesis would consequently appear as a positive relationship between model toxicity and imperfect mimicry, with imperfect mimics occurring with highly toxic models (Goodale & Sneddon, 1977; Lindström *et al*., 1997).

Polymorphism

While some species are typified by a universal phenotype, many are phenotypically diverse. When this phenotypic diversity is discontinuous in nature, the species is termed 'polymorphic.' Color polymorphism has long been studied to understand evolutionary processes between predators and prey, and in many cases selection generating polymorphisms is visually-mediated by predators (reviewed in Bond, 2007). For instance, land snails of the genus *Cepaea* are remarkably polymorphic, with different color morphs found in distinct environments that are maximally camouflaged where found, presumably as a result of selection by visual predators (Cain & Sheppard, 1954). Likewise, different color morphs of the moth *Biston betularia* have been selectively favored as a consequence of varying pollution levels during and following the Industrial Revolution (Grant, 1999).

In mimicry systems, polymorphism is a regular occurrence, with mimics from different populations often resembling local models (Brown & Benson, 1974; Greene & McDiarmid, 1981; Darst & Cummings, 2006). Less frequently, color morphs in mimicry systems are divided between mimetic and non-mimetic morphs within populations. In such cases, mimics are usually restricted to a single sex (Kunte, 2009). Even more rare, mimic/non-mimic polymorphism may be found in both sexes within populations (Tilley *et al.*, 1982). Mimic/non-mimic polymorphism is predicted in some cases because of the inherently frequency-dependent nature of Batesian mimicry, where mimics are most fit when

they are rarely encountered relative to their unpalatable models (Turner, 1977; Turner, 1978; Speed & Ruxton, 2010). These examples of mimic/non-mimic polymorphism present a unique opportunity to study the selective benefits of mimic and non/mimic phenotypes while holding constant other aspects of phenotype and life history.

Multiple Predators and Mimicry

Predation pressure on a population is often not limited to a single predator species (Endler, 1986). Any discussion of mimic evolution thus benefits from a consideration of multiple predators, though this is rarely done (Pekár *et al*., 2011). Importantly, multiple predators may select for mimicry in a similar fashion, or additional predators may impose selection on prey color and pattern differently from mimicry, thereby influencing the evolution of prey phenotype. For example, foraging predators may identify and target prey whose coloration has greater contrast with the background environment (i.e. more conspicuous), thereby driving the evolution of prey difficult for predators to initially detect (Endler, 1978). Predators may also recognize, and then attack, those prey that are most common in the environment, resulting in frequency-dependent selection against common prey (Allen, 1988). On the other hand, predators may avoid unfamiliar prey (called 'dietary conservatism'; Marples *et al*., 2007), or predators may not use coloration or patterning in any way during foraging, perhaps instead utilizing other senses, such as olfaction (Endler, 1986; Ruxton, 2009; Hughes *et al.*, 2010). Importantly, these hypotheses are not necessarily exclusive. Predators likely incorporate several of the above strategies into a single composite behavior when responding to color cues in potential prey (Endler, 1986).

Salamanders as a Mimicry System

The salamanders *Notophthalmus viridescens* and *Plethodon cinereus* present an appealing group with which to test predictions of Batesian mimicry in the wild. *Notophthalmus viridescens,* a widespread salamander in eastern North America, has a triphasic life cycle, where aquatic larvae often transform into a terrestrial juvenile (eft) stage, which then matures into an aquatic newt (Petranka, 1998). Efts of *N. viridescens* are strikingly red-orange in coloration (Fig. 1A), and contain tetrototoxin (TTX) in their skin, which is noxious to most potential predators (Brodie, 1968; adult newts can also contain TTX: Yotsu-Yamashita & Mebs 2003). Terrestrial *N. viridescens* efts are frequently avoided in feeding experiments using avian predators (e.g. Brodie, 1968; Hurlbert, 1970; Tilley *et al*., 1982), which is presumably due to the unpalatability conferred by tetrodotoxin and their distinctive coloration as a signal of unpalatability (Hurlbert, 1970). Thus, red-orange coloration in *N. viridescens* is likely a conspicuous warning signal to avian predators, thereby making *N. viridescens* an aposematic species with bright warning coloration and noxious defensive chemicals.

By contrast, *Plethodon cinereus,* whose geographic distribution overlaps broadly with that of *N. viridescens*, is not aposematic. This species displays three color-morphs; the two most common are the unstriped morph (Fig. 1C) and the striped morph (Fig. 1D), which are typically not considered conspicuous (Petranka, 1998). However, in some localities a third red-orange morph (erythristic: Fig. 1B) is found that appears similar in coloration to *N. viridescens.* Unlike *N. viridescens*, *P. cinereus* is not noxious and does not contain tetrodotoxin (Brodie & Brodie. 1980; Tilley *et al*., 1982). However, because the two species can be found sympatrically, and because of their similar coloration, the erythristic morph of

P. cinereus is thought to be a Batesian mimic of *N. viridescens* (Lotter & Scott, 1977). Lending support to this hypothesis, two empirical field studies have found that wild and captive bird predators avoid *N. viridescens* and erythristic *P. cinereus* similarly, while they readily consume striped *P. cinereus* (Brodie & Brodie, 1980, Tilley *et al*., 1982). Thus, behavioral studies (e.g. Brodie & Brodie, 1980, Tilley *et al*., 1982) and toxicity assays (e.g. Brodie, 1968) provide evidence consistent with the hypothesis of Batesian mimicry in this system, and suggest that bird predators may select salamander prey based on their coloration and toxicity.

Dissertation Outline

A complex relationship between predators and their prey governs the evolution of Batesian mimicry. In this dissertation, I examine the evolution of such a relationship between the salamanders *Plethodon cinereus* and *Notophthalmus viridescens*. In Chapter 2, I evaluate how several vertebrate predator groups may perceive mimicry between *P. cinereus* and *N. viridescens.* In Chapter 3, I look for evidence of selection on model phenotype in this system. In Chapter 4, I identify whether variation in model unpalatability can influence the evolution of mimicry in *P. cinereus*. I shift perspective in Chapter 5 to understand how a complex predator community consisting of multiple species may alter predictions of phenotype evolution in *P. cinereus* by quantifying mammalian predation on salamander clay replicas. Finally, the results from earlier chapters are summarized in Chapter 6 to discuss the evolution of mimicry between *P. cinereus* and *N. viridescens*.

Figure 1. Study system. A) *Notophthalmus viridescens* eft (model), B) erythristic *Plethodon cinereus* (mimic), C) unstriped *P. cinereus*, D) striped *P. cinereus.*

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CHAPTER II

PREDATOR PERCEPTION OF BATESIAN MIMICRY AND CONSPICUOUSNESS IN A SALAMANDER

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Abstract

In Batesian mimicry a palatable mimic deceives predators by resembling an unpalatable model. The evolution of Batesian mimicry relies on the visual capabilities of the potential predators, as prey detection provides the selective force driving evolutionary change. We compared the visual capabilities of several potential predators to test predictions stemming from the hypothesis of Batesian mimicry between two salamanders: the model species *Notophthalmus viridescens*, and polymorphic mimic, *Plethodon cinereus*. First, we found mimicry to be restricted to coloration, but not brightness. Second, only bird predators appeared able to discriminate between the colors of models and non-mimic *P. cinereus*. Third, estimates of salamander conspicuousness were background-dependent, corresponding to predictions only for backgrounds against which salamanders are most active. These results support the hypothesis that birds influence the evolution of Batesian mimicry in *P. cinereus*, as they are the only group examined capable of differentiating *N. viridescens* and non-mimetic *P. cinereus*. Additionally, patterns of conspicuousness suggest that selection from predators may drive the evolution of conspicuousness in this system. This study confirms the expectation that the visual abilities of predators may influence the evolution of Batesian mimicry, but the role of conspicuousness may be more complex than previously thought.

Introduction

Batesian mimicry, in which a palatable species resembles an unpalatable model species in its phenotype or behavior, has long been recognized as an example of adaptation and convergent evolution (Ruxton *et al*., 2004). This form of mimicry first requires that the unpalatable model provide a warning signal detectable by potential predators that indicates significant costs to consuming it as prey. Frequently, this warning signal is visual in nature (e.g., conspicuous coloration: Rowe and Guilford, 2000). Second, the mimic must copy the model's signal to deceive potential predators. Importantly, both the warning signal and subsequent mimicry depend upon the visual capabilities of potential predators, as predators are the selective agents in this system.

Observations of Batesian mimicry have stimulated considerable interest among ecologists and evolutionary biologists (e.g., Bates, 1862; Edmunds, 1969; Greene and McDiarmid, 1981), and theoretical and empirical work on both predators and their prey have led to many predictions of interspecific interactions in Batesian mimicry systems (e.g., reviewed in Mallet and Joron, 1999; Speed, 1999; Ruxton *et al*., 2004; Pfennig and Mullen, 2010; Stoddard, 2012). In particular, three predictions of warning signal design are expected in most systems of Batesian mimicry. First, mimics are predicted to generally match model species in phenotype (Ruxton *et al*., 2004). In particular, because predators are driving the evolution of mimicry, Batesian mimics are predicted to resemble their models in ways that potential predators can perceive (e.g. Kikuchi and Pfennig, 2010). Second, unpalatable models are often predicted to be conspicuous and therefore aposematic (Roper and Redston, 1987; Ruxton *et al*., 2004; Mappes *et al*., 2005; but see Wüster *et al*., 2004). When this is the case, mimics are predicted to evolve conspicuousness as well (reviewed in Ruxton *et al*.,

2004 and discussion in Franks *et al.,* 2009). Finally, since conspicuousness is perceived as the degree of contrast between a target and its background (Ruxton *et al*., 2004), different backgrounds may influence prey detectability (e.g. Siddiqi *et al.,* 2004; Macedonia *et al*., 2009; Willink *et al*., 2013). Therefore, the patterns of conspicuousness predicted between models and mimics may be background-specific. The evolution of Batesian mimicry has been extensively studied in numerous taxa (e.g. Tilley *et al*., 1982; Pfennig *et al.,* 2001; Kunte, 2009), and some of these studies have examined the visual capabilities of potential predators to test predictions of mimicry (e.g. Cheney & Marshall, 2009; Papadopulos *et al*., 2013). However, to date the role of conspicuousness in the evolution of Batesian mimicry remains under-examined, particularly from the perspective of potential predators.

The salamanders *Notophthalmus viridescens* and *Plethodon cinereus* present an appealing group with which to test predictions of Batesian mimicry in the wild. *Notophthalmus viridescens,* a widespread salamander in Eastern North America, has a triphasic life cycle, where aquatic larvae often transform into a terrestrial juvenile (eft) stage, which then matures into an aquatic newt (Petranka, 1998). Efts of *N. viridescens* are strikingly red-orange in coloration (Fig. 1C), and contain tetrototoxin (TTX) in their skin, which is noxious to most potential predators (Brodie, 1968; adult newts can also contain TTX: Yotsu-Yamashita and Mebs, 2003). Terrestrial *N. viridescens* efts are frequently avoided in feeding experiments using vertebrate predators (e.g. Brodie, 1968; Hurlbert, 1970; Tilley *et al*., 1982), which is presumably due to the unpalatability conferred by tetrodotoxin and their distinctive coloration as a signal of unpalatability (Hurlbert, 1970). Thus, red-orange coloration in *N. viridescens* is likely a conspicuous warning signal to predators,

thereby making *N. viridescens* an aposematic species with bright warning coloration and noxious defensive chemicals.

By contrast, *Plethodon cinereus,* whose geographic distribution overlaps broadly with that of *N. viridescens*, is not aposematic. This species displays three color-morphs; the two most common are the striped morph (Fig. 1A) and the unstriped morph, which are typically not considered conspicuous (Petranka, 1998). However, in some localities a third red-orange morph (erythristic: Fig. 1B) is found that appears similar in coloration to *N. viridescens.* Unlike *N. viridescens*, *P. cinereus* is not noxious and does not contain tetrodotoxin (Brodie & Brodie, 1980; Tilley *et al*., 1982). However, because the two species can be found sympatrically, and because of their similar coloration, the erythristic morph of *P. cinereus* is thought to be a Batesian mimic of *N. viridescens* (Lotter & Scott, 1977). Lending support to this hypothesis, two empirical field studies have found that wild and captive bird predators avoid *N. viridescens* and erythristic *P. cinereus* similarly, while they readily consume striped *P. cinereus* (Brodie & Brodie, 1980; Tilley *et al*., 1982). Thus, behavioral studies (e.g. Brodie & Brodie, 1980; Tilley *et al*., 1982) and toxicity assays (e.g. Brodie, 1968) provide evidence consistent with the hypothesis of Batesian mimicry in this system, and suggest that bird predators may select salamander prey based on their coloration and toxicity.

In this study we tested three predictions of the hypothesis of Batesian mimicry between *N. viridescens* and *P. cinereus.* First, we evaluated whether erythristic *P. cinereus* and the eft stage of *N. viridescens* are similar in color and brightness, particularly from the perspective of potential predators. Second, we tested whether *N. viridescens,* and subsequently *P. cinereus*, are conspicuous to potential predators. Third, we determined whether different natural backgrounds could affect estimates of conspicuousness for each predator.

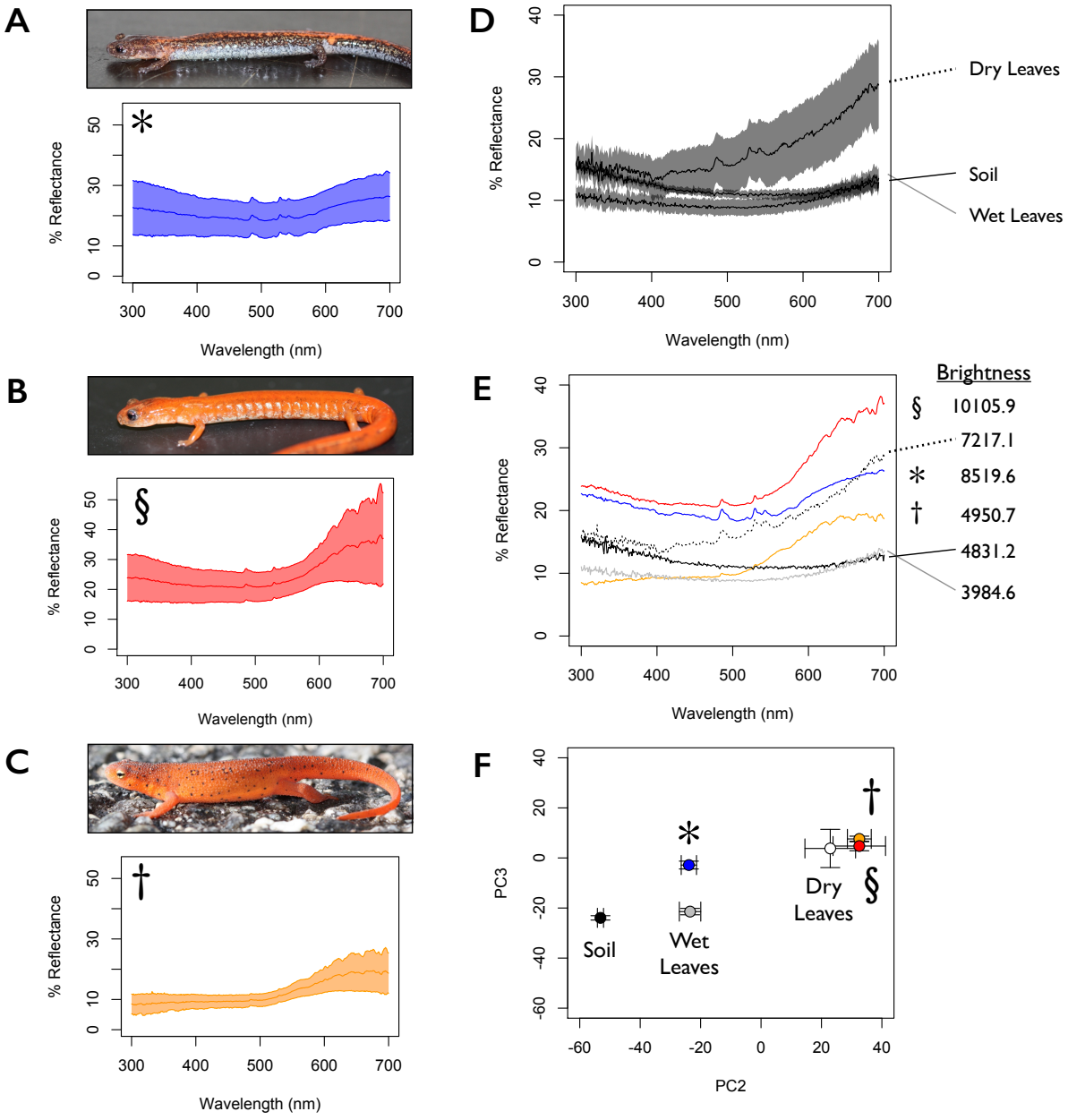

Figure 1. Panels A-C: representative salamanders and their spectra, with striped *P. cinereus* (A; *), erythristic *P. cinereus* (B; §), and *N. viridescens* (C; †). Shaded regions in each plot correspond to the standard deviation of spectra for each group. Panel D: background spectra used in the analyses. Panel E: mean spectra for each salamander and background group, with mean brightness for each group to the right of each spectrum. Panel F: Multivariate coloration of each salamander and background group. Least squares means and standard error bars for each group are plotted.

In particular, we identified if such variation could facilitate identification of the predators driving selection for color similarity and under which conditions they do so. Although birds are hypothesized to generate selection that may drive the evolution of Batesian mimicry in this system (Brodie & Brodie, 1980; Tilley *et al*., 1982), several vertebrate predators are also implicated as natural salamander predators (including UV-sensitive birds: Lotter & Scott, 1977; Fenster & Fenster, 1996; snakes: Arnold, 1982; and mammals: Brodie *et al*., 1979). We therefore used visual models generated for multiple potential predator classes and examined conspicuousness and similarity between *N. viridescens* and *P. cinereus* with respect to the visual capabilities of three predator groups relative to three potential backgrounds. This analytical framework allows us to determine not only if erythristic *P. cinereus* appear as mimics of *N. viridescens* to potential vertebrate predators on the basis of color or brightness, but also whether salamander conspicuousness is dependent on the background against which it is viewed.

Materials and Methods

Salamander Collection and Coloration Quantification

In summer 2011 we obtained salamanders from 14 sites in western Massachusetts where both species are widely distributed. In this region the erythristic morph of *P. cinereus* is uncommon but is sympatric with *N. viridescens* more often than expected by chance ($P = 0.0342$; Appendix A). We collected a total of 521 adult *P. cinereus* and eft-stage *N. viridescens* for field measurements of coloration (N = 123 erythristic *P. cinereus*, 281 striped *P. cinereus*, and 117 *N. viridescens;* unstriped *P. cinereus* were not present at these sites). All individuals were first anesthetized using tricaine methanesulfonate (MS-222: *P. cinereus*) or

by applying benzocaine to the ventral surface of the head (*N. viridescens*). There is a possibility that differences in anesthetization could result in uneven changes in coloration between salamander groups. However, such physiologically-based color changes have only been observed in an unrelated salamander lineage (Garcia *et al*., 2003), and we did not observe any physiological color-change in our salamanders. We then measured spectral reflectance from a single point in the mid-dorsal region of each salamander using a portable JAZ-PX spectrometer (OceanOptics, Dunedin, FL) with a 100 μm entrance slit, a pulsed xenon lamp, and a QR400-7-UV-BX reflectance probe. This probe was fitted with a tip that restricted the measured patch to a 2 mm diameter circle, maintained a constant distance (20 mm) between probe tip and measured patch, and excluded ambient light. We held the probe perpendicular to the surface of the salamanders and used a Spectralon white reflectance standard between each animal to correct for drift in lamp intensity (see e.g., Kraemer *et al*., 2012). Measuring reflectance at a perpendicular angle can introduce specular glare, particularly when coloration is influenced by structural pigmentation in the target animal (Endler, 1990). However, the dorsal coloration of these salamanders is pigment-based, with limited structural elements (Bagnara & Taylor, 1970), which in this case, minimized the potential for specular glare. We measured each spectrum at 1nm intervals from 300 to 700 nm. We acknowledge that color variation among sites may be present. However, insufficient sample sizes within sites precluded a formal analysis of the multivariate color variation among geographic sites and within morphs (Appendix A). Therefore, only comparisons among morphs were performed. From one site where salamanders were collected (Lilly Pond Wildlife Management Area, Goshen, Massachusetts) we obtained 10 reflectance spectra each of naturally wet and dry leaf litter on 9 June 2012. We also collected 20 spectra of bare soil

from a surrogate locality (Pammel Woods, Ames, Iowa; 11 July 2013) to represent backgrounds that predators might view salamanders against (Fig. 1B). We acknowledge that sampling of background spectra is somewhat limited. However, all sites consisted of a similar compliment of hemlock-northern hardwood forest, and thus we assumed that the backgrounds against which salamanders were viewed did not vary among sites.

Multivariate Brightness and Color Quantification

To obtain a set of color and brightness variables for statistical comparison (*sensu* Endler & Thery, 1996; Grill & Rush, 2000) we performed principal components analysis on the raw reflectance spectra. In this representation, PC1 is interpreted as brightness, or the total amount of light reflected from the measured patch, while the remaining PC-axes describe aspects of chroma and hue (henceforth coloration: see Endler, 1990; Endler & Thery, 1996; Grill & Rush, 2000). In our case, PC1 was highly correlated with an independent measure of overall brightness (Q_t; *sensu* Endler, 1990; $r > 0.99$). The remaining principal component axes (PC_2 - PC_{401}) describe the shape of the spectral curve and thus the color that each spectrum represented (Grill & Rush, 2000). For our analyses we used Q_t, or the total reflectance under each spectrum, to evaluate brightness and PC_2 - PC_{401} to evaluate coloration.

Visual model

We estimated the ability of potential predators to discriminate between various prey items and typical background surfaces with a visual model developed by Vorobyev *et al.* (2001). This analytical model is derived from the signal to noise ratio of predator

photoreceptors, and assumes that the ability of predators to discriminate between objects depends on this ratio (Vorobyev *et al*., 1998). The model yields estimates of visual contrast between a target and a background (as in Maan & Cummings, 2012) or as a contrast between two targets (as in Siddiqi *et al*., 2004). These contrasts are calculated for each of two visual channels, chromatic (ΔS) and achromatic (ΔL), in which the chromatic visual channel summarizes the aspects of visual stimuli pertaining to coloration (i.e. chroma and hue), while the achromatic visual channel summarizes the aspects of visual stimuli pertaining to brightness. In each case, large contrast values correspond to clear and discriminable differences between targets and backgrounds (or between targets) from the perspective of the predator, while smaller values represent similar coloration between targets and backgrounds (or between targets) and are thus potentially indistinguishable. Contrast values of 1 or smaller are thought to describe indistinguishable pairs of spectra because photoreceptor processing of visual stimuli will result in more noise than original signal, while values greater than 1 correspond to more signal than noise (Vorobyev *et al*., 1998).

As input data, the visual model requires reflectance measures of the target and background, background habitat irradiance, and photoreceptor sensitivities of the predator. We used spectral data obtained for each salamander as described above. Average wet leaf litter, dry leaf litter, and bare soil spectra served as backgrounds. We used two irradiance measures: the standard D65 daylight irradiance (Judd *et al*., 1964) and a forest shade irradiance measure reported elsewhere (Maan & Cummings, 2009). We report only the contrasts calculated with the forest irradiance because the relationships among contrasts did not differ as a result of irradiance measurement.

Three classes of vertebrates have been implicated as potential predators of North American terrestrial salamanders, and differ substantially with respect to their visual capabilities. While we were unable to estimate the spectral sensitivities of specific salamander predator species for this system, we were able to use known spectral sensitivities of related vertebrates to compare the visual capabilities for each predator class: birds (salamander predators: tetrachromatic blue jays and brown thrashers, Howard & Brodie, 1973, Chen & Goldsmith, 1986 – visual model: the tetrachromatic blue tit, Hart *et al*., 2000), snakes (salamander predators: garter snakes, Arnold, 1982 – visual model: eastern garter snake, Sillman, 1997), and mammals (salamander predators: shrews and other small, opportunistic mammals, Brodie *et al*., 1979, Petranka, 1998 – visual model: thirteen-lined ground squirrel, Jacobs *et al*., 1985). We used a rhodopsin template (based on vitamin A1; Govardovskii *et al*., 2000) to estimate the spectral sensitivities for the garter snake (*Thamnophis sirtalis*), with photoreceptor peak sensitivities of: long-wave sensitive (LWS) λ_{max} = 554, short-wave sensitive (SWS) λ_{max} = 482, and UV-wave sensitive (UWS) λ_{max} = 360 (Sillman *et al*., 1997). We used the same rhodopsin template to estimate spectral sensitivities for the thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*), which is an opportunistic, diurnal forager with LWS λ_{max} = 517 and SWS λ_{max} = 437 (Jacobs *et al*., 1985). A full description of the visual model calculations and corresponding code are found in Appendix A. All analyses were conducted in R 2.15 (R Development Core Team 2012).

Statistical Analyses

Prediction 1a: Model-mimic color and brightness similarity

To evaluate the similarity of warning signals predicted for Batesian mimicry, we compared predator-independent estimates of color (PC-color data) and brightness (Q_t values as estimated by integrating across each spectrum from 300-700 nm) across salamander groups (eft *N. viridescens,* striped *P. cinereus,* erythristic *P. cinereus*) and background groups (wet leaf litter, dry leaf litter, soil) using Permutational-MANOVA (for color data; Anderson, 2001) or analysis of variance (ANOVA; for brightness data). Permutational-MANOVA was used because of the high-dimensionality of the color space. We then evaluated pairwise differences between groups using randomization. Briefly, the observed pairwise differences between groups (measured as Euclidean distances) were first obtained from the least-squares (LS) means for each group. Specimens were then permuted with respect to group identity and the model was recalculated (permutations = 999), and pairwise distances between LS means were obtained. The proportion of randomly generated distances greater than the observed was then treated as the significance of each pairwise comparison (for statistical details see Anderson & Ter Braak, 2003; Adams & Collyer, 2009; Adams, 2010; Adams & Nistri, 2010). A sequential Bonferroni correction was used to account for multiple testing during pairwise comparisons.

Prediction 1b: Salamander Discriminability

For each predator visual model, we used a one-sample t-test to evaluate color and brightness contrasts obtained for *P. cinereus* relative to *N. viridescens*. Contrast scores significantly greater than 1.0 suggest that the predator can differentiate between these two

salamanders. This analysis was repeated for brightness and color components of each *P. cinereus* morph and for each predator model. A sequential Bonferroni correction was used to account for multiple testing during t-tests.

Predictions 2 and 3: Salamander conspicuousness

We also compared salamander color and brightness to various backgrounds using a one-sample t-test. Contrast scores significantly greater than 1.0 suggest that salamanders are conspicuous and therefore visible to the predator against that background. This analysis was repeated for each salamander group against each background and for each predator model. Additionally, we compared the conspicuousness of *P. cinereus* color-morphs using two-sample t-tests. A sequential Bonferroni correction was used to account for multiple testing during t-tests. Statistical analyses evaluating all predictions were performed in R 2.15 (R Development Core Team, 2012).

Results

Prediction 1a: Model-mimic color similarity

We found significant differences in brightness among salamander and background groups ($F = 90.3$, $P < 0.001$). Pairwise comparisons revealed that erythristic *P. cinereus* were brighter than all other groups, while *N. viridescens* was similar in brightness to all backgrounds examined (Table 1A: Fig. 1E). Additionally, striped *P. cinereus* were not different in brightness from dry leaf litter. Using Permutational-MANOVA we also found large and significant differences in overall coloration among salamander and background groups ($F = 2.127$, $P < 0.001$). Pairwise comparisons revealed that the coloration of both *N.*

viridescens and erythristic *P. cinereus* differed significantly from striped *P. cinereus* and soil. Additionally, dry leaf litter and soil coloration were significantly different (Table 1B: Fig. 1F). All other pairwise comparisons between groups were non-significant.

Prediction 1b: Salamander discriminability

Based on the visual models, both *P. cinereus* morphs were significantly different from *N. viridescens* in brightness from the perspective of all three predators (results not shown). However, the visual models indicated that neither mammals nor snakes could differentiate between the coloration of *N. viridescens* and either *P. cinereus* morph. Importantly, while birds could differentiate between *N. viridescens* and striped *P. cinereus* coloration, they were unable to differentiate between *N. viridescens* and erythristic *P. cinereus* (Fig. 2A).

Table 1. Differences between salamander and background groups in (A) brightness and (B) color. Values below the diagonal are the Euclidean distances between least squares means, while values above the diagonal are the pairwise P-values from randomization. 'Erythristic' and 'Striped' denote respective color morphs of *P. cinereus*, and '*N.v.*' denotes *N. viridescens.* Bold values indicate significance.

A	*N.v.*	Erythristic	Striped	Wet Leaves	Dry Leaves	Soil
N.v	-	**0.001**	**0.001**	0.294	0.021	0.860
Erythristic	**5155.24**	-	**0.001**	**0.001**	**0.003**	**0.001**
Striped	**3568.83**	**1586.41**	-	**0.001**	0.038	**0.001**
Wet Leaves	966.11	**6121.35**	**4534.94**	-	0.013	0.459
Dry Leaves	2266.34	**2888.90**	1302.49	3232.45	-	0.038
Soil	119.59	**5274.83**	**3688.42**	846.52	2385.93	-
B	*N.v.*	Erythristic	Striped	Wet Leaves	Dry Leaves	Soil
N.v.	-	0.477	**0.001**	0.011	0.218	**0.001**
Erythristic	6.92	-	**0.001**	0.012	0.276	**0.001**
Striped	**57.90**	**56.99**	-	0.300	0.021	0.018
Wet Leaves	**64.67**	**62.66**	21.29	-	0.061	0.231
Dry Leaves	26.13	23.84	**51.44**	56.52	-	**0.003**
Soil	**92.03**	**90.83**	**37.59**	30.84	**84.11**	-

Predictions 2 and 3: Salamander conspicuousness

Using the visual models for potential predators we found that all salamander groups were conspicuous against all backgrounds with respect to brightness (results not shown). By contrast, when viewed against dry leaf litter, all salamander groups were conspicuous in their coloration from the perspective of birds, while only striped *P. cinereus* were conspicuous in coloration for snakes. No salamander groups were conspicuous in coloration from the perspective of mammals (Fig. 2B). Against both soil and wet leaf litter, *N. viridescens* coloration was conspicuous to all three predators (Fig. 2C,D). Against soil both striped and erythristic *P. cinereus* coloration were conspicuous to birds (Fig. 2C). Erythristic *P. cinereus* brightness was significantly more conspicuous than striped *P. cinereus* against wet leaf litter and soil for all predators (Table 2A,B). Against dry leaf litter, erythristic *P. cinereus* brightness was significantly more conspicuous than striped *P. cinereus* for birds (Table 2C). Finally, for mammals against dry leaf litter and soil and for snakes against dry leaf litter, striped *P. cinereus* coloration was more conspicuous than erythristic *P. cinereus* coloration (Table 2B,C).

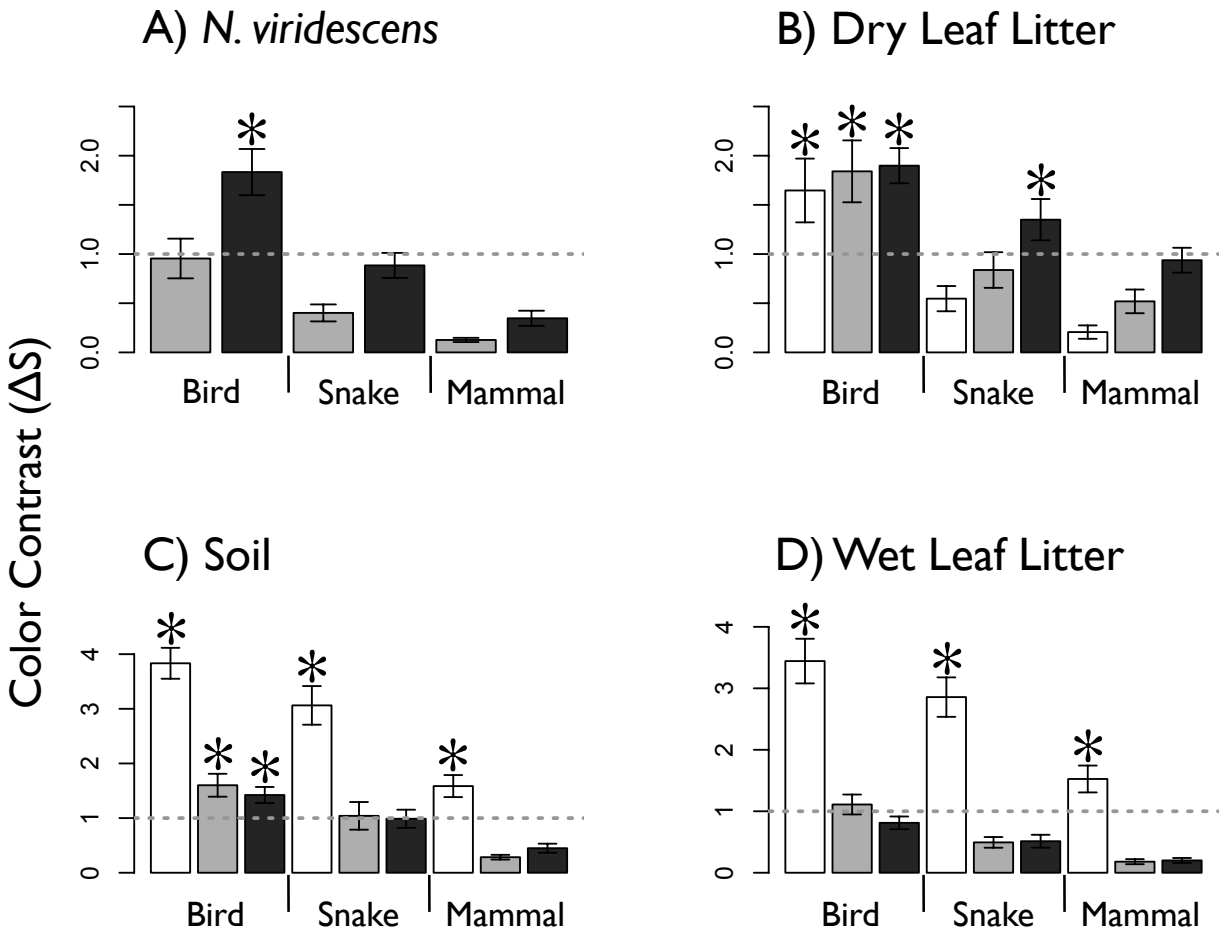

Figure 2. Salamander color contrasts as predicted for each group from predator visual models. Contrasts were estimated against A) *N. viridescens*, B) dry leaf litter, C) soil, and D) wet leaf litter. Contrasts predicted to be distinguishable lie above the horizontal dotted line and are denoted with an asterisk. White bars correspond to *N. viridescens*, light gray bars to erythristic *P. cinereus*, and dark gray bars to striped *P. cinereus*.

Table 2. Color and brightness differences in conspicuousness between erythristic and striped *P. cinereus* against (A) wet leaf litter, (B) dry leaf litter, and (C) soil. Bold values indicate significant differences.

A		*t*	df	*P*-value
Bird viewer	Color	3.05	225.4	0.003
	Brightness	**10.45**	**329.4**	**<0.001**
Snake viewer	Color	-0.27	382.4	0.786
	Brightness	**6.74**	**328.5**	**<0.001**
Mammal viewer	Color	-0.69	334.2	0.493
	Brightness	**5.40**	**329.1**	**<0.001**
B		*t*	df	*P*-value
Bird viewer	Color	-0.31	204.2	0.755
	Brightness	**5.55**	**205.4**	**<0.001**
Snake viewer	**Color**	**-3.61**	**375.2**	**<0.001**
	Brightness	2.42	259.5	0.016
Mammal viewer	**Color**	**-4.69**	**352.6**	**<0.001**
	Brightness	1.10	228.5	0.271
C		*t*	df	*P*-value
Bird viewer	Color	1.37	246.4	0.172
	Brightness	**10.47**	**347.1**	**<0.001**
Snake viewer	Color	0.34	230.7	0.731
	Brightness	**7.38**	**341.4**	**<0.001**
Mammal viewer	**Color**	**-3.45**	**387.1**	**<0.001**
	Brightness	**5.12**	**328.9**	**<0.001**

Discussion

How predators perceive and classify Batesian mimics and their models is of central importance to understanding mimicry. In this study we used a visual model to test three predictions of Batesian mimicry from the perspective of predators. First, we found models (*N. viridescens*) and mimics (erythristic *P. cinereus*) to be similar in coloration while dissimilar in brightness. Second, models and mimics were indistinguishable in coloration from the perspective of all three vertebrate predators, and only birds appeared able to discriminate between models and non-mimic (striped) *P. cinereus* on the basis of coloration. Third, *N. viridescens* coloration was more conspicuous than either *P. cinereus* morph when viewed against both wet leaf litter and soil for all predators, but not when viewed against dry leaf litter. Finally, erythristic *P. cinereus* were more conspicuous than striped *P. cinereus* under most conditions for brightness, but not for coloration.

Model – Mimic Color Similarity

The finding that the coloration of *N. viridescens* and erythristic *P. cinereus* did not differ statistically is not surprising, as similarity of color between the two is what initially prompted biologists to suggest erythristic *P. cinereus* were Batesian mimics of *N. viridescens* (Lotter & Scott, 1977). Conversely, significant differences in brightness between *N. viridescens* and erythristic *P. cinereus* were unforeseen and suggest that mimicry in this system is restricted to coloration and not brightness, as these species are most similar in coloration but not brightness. Interestingly, *P. cinereus* are not the only mimics of *N. viridescens*. *N. viridescens* may also serve as the model for three additional salamander species (*Pseudotriton ruber*: Howard & Brodie, 1971; *Gyrinophilus porphyriticus*: Howard

& Brodie, 1973*; Pseudotriton montanus*: Huheey & Brandon, 1974) and a leech (*Macrobdella diploteria*: McCallum *et al*., 2008). Further research that disentangles the elements of warning signal design (i.e. color, brightness, and pattern) in these species may shed light on how mimicry evolves in this system and why some aspects of warning signals are mimicked while others are not.

Salamander Discriminability

Our results show that when viewed from the perspective of predators, models and mimics were quite similar in coloration, though dissimilar in brightness, indicating that vertebrate predators likely classify erythristic *P. cinereus* as mimics of *N. viridescens* on the basis of coloration and not brightness. Additionally, we found that snakes and mammals appear incapable of differentiating between the coloration of *N. viridescens* and non-mimic *P. cinereus*. This suggests that while these predators prey on salamanders (Brodie *et al*., 1979; Arnold, 1982), they do not view erythristic *P. cinereus* as mimics of *N. viridescens*. Conversely, bird predators are able to differentiate between the coloration of *N. viridescens* and striped, non-mimic *P. cinereus*, but are not capable of distinguishing between *N. viridescens* and erythristic *P. cinereus*. These observations imply that birds view erythristic *P. cinereus* as being similar in coloration to *N. viridescens*, and thus, the model to mimic relationship is preserved when considering bird predators. We note however that our results rely on the manner in which the light environment (i.e. irradiance) is estimated, and as such are somewhat preliminary in scope because we used a single irradiance measure in our analyses. Indeed, variation in the light environment by site, time of day, and season can alter salamander discriminability by predators (for a review of the effect of variation in light

environment on animal vision, see Endler, 1993), but the influence of such variation is currently unknown for mimicry systems. Nevertheless, our preliminary findings lend support to the hypothesis that birds are the dominant selective force driving the evolution of mimicry in *P. cinereus* (Brodie & Brodie, 1980; Tilley *et al*., 1982). Visually hunting and diurnal litter-searching birds, such as thrushes and robins, are known to search for and consume salamanders (e.g. Coker, 1931; Wilson & Simon, 1985). By searching through litter during the day, birds are likely to expose individuals of both salamander species on a regular basis. The ability of birds to differentiate between *N. viridescens* and striped *P. cinereus,* but not between *N. viridescens* and erythristic *P. cinereus,* may thus provide the erythristic morph with a selective advantage over striped individuals, thereby helping to maintain erythristic individuals in *P. cinereus* populations.

Salamander Conspicuousness

Since *N. viridescens* is unpalatable to predators (Brodie, 1968), its red-orange coloration may serve as a conspicuous warning signal to predators. Conspicuousness is commonly assumed for defended model species of Batesian mimics (Ruxton *et al*., 2004; but see Wüster *et al*., 2004). Consistent with this prediction, we found that *N. viridescens* were conspicuous for all three vertebrate predator types examined. However, they were most prominent when viewed against wet leaf litter and soil, while against dry leaf litter, *N. viridescens* coloration was no more conspicuous than that of *P. cinereus*. This result may appear puzzling, until one considers the activity periods of terrestrial salamanders. Salamanders like *N. viridescens* and *P. cinereus* are most active, and thus likely to encounter predators, when the environment is cool and wet (Petranka, 1998). Consequently, selection

should drive *N. viridescens* to appear most conspicuous against wet, and not dry, leaf litter. Our data are consistent with this prediction, as *N. viridescens* were most conspicuous against wet leaf litter and soil. We note that this hypothesis could be tested by identifying the conditions in which *N. viridescens* and *P. cinereus* experience the strongest selection pressure from predators, for example, through studies that expose clay salamander replicas to predators under various environmental conditions (e.g. *sensu* Brodie, 1993; Pfennig *et al*., 2001; Kuchta, 2005).

In contrast to *N. viridescens*, *P. cinereus* coloration was generally not conspicuous to predators. This result is surprising, as it implies that erythristic *P. cinereus* coloration is less conspicuous than *N. viridescens* coloration. This result is also potentially important, as it is commonly assumed that the evolution of Batesian mimicry necessitates mimics to overcome the 'cost of conspicuousness,' in which mimicking a conspicuous species requires mimics to adopt a conspicuous phenotype, which should increase their exposure to predators (Speed & Ruxton, 2010). Our results suggest that *N. viridescens* may be more visible to predators than erythristic *P. cinereus*. The ability to mimic defended species while simultaneously appearing less conspicuous to predators may make Batesian mimicry more likely to evolve than conditions in which the mimic is as conspicuous as the defended model (Sherratt & Franks, 2005). The ability to mimic *N. viridescens* while remaining relatively inconspicuous may have contributed to the evolution of Batesian mimicry in this species. Interestingly, erythristic *P. cinereus* were brighter than striped *P. cinereus* (Table 1; Fig. 1). Color and brightness are tightly linked (Duellman & Trueb, 1986), perhaps constraining the evolution of brightness in this species. Consequently, there may be conspicuous costs for erythristic *P. cinereus* apart from coloration.

Conclusions

Batesian mimicry requires mimics to resemble unpalatable models, particularly from the perspective of natural predators, and this resemblance may require mimics to appear conspicuous to predators. Here we provide an explicit test of three predictions of mimicry in a system consisting of two salamander species. We found mimics and models to be similar in overall coloration and from the perspective of potential predators. Additionally, unpalatable models were found to be conspicuous to predators, while mimics may appear less conspicuous, particularly under the conditions that they are more likely to encounter predators, which supports the hypothesis that predators can drive the evolution of conspicuousness in prey species. These findings also indicate that while conspicuousness may be an important factor for the evolution of mimicry, other factors likely influence how predators perceive conspicuousness. By examining the impact of conspicuousness in natural systems we will better understand why mimicry evolves in some species but not others.

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Appendix A

Visual Model Calculations

The first step in the visual model is to calculate each photoreceptor's quantum catch (Q_c) for each target or background spectra by multiplying the light environment, target or background reflectance, and photoreceptor sensitivity at each wavelength:

$$Q_c = \int_{\lambda=300}^{700} I(\lambda)R(\lambda)A_c d\lambda,$$

Next, Q_c is adjusted for the achromatic background light environment via the von Kries transformation: $q_c = k_c Q_c$, where

$$k_c = \frac{1}{\int_{\lambda=300}^{700} I(\lambda)A_c d\lambda}.$$

The von Kries transformation ensures color constancy between different light environments and is thought to be an essential step in animal visual processing (reviewed by Neumeyer, 1998).

Photoreceptor signals follow the Weber-Fetchner Laws, which assume that the limit of discrimination between two stimuli is proportional to the logarithm of their differences (Vorobyev *et al*., 1998). Subsequently, the contrast between target and background or between two targets for each photoreceptor is calculated as $\Delta f_c = \ln [q_c(\text{target}_1) - q_c(\text{target}_2)]$.

To estimate the impact of photoreceptor noise (ω) on the discriminability of different target spectra, noise is assumed to decrease as the number of receptor types in the retina (η)

increases such that $\omega = \nu/\eta$, where ν is the Weber fraction for each receptor type (as in Vorobyev *et al*., 1998). The Weber fraction (or Fechner fraction) is defined as the constant to which discriminability scales in relation to the background irradiance. We used the Weber fraction of an avian LWS cone, $\nu = 0.10$ (Maier, 1992), for the avian visual model. For the snake we used $\nu = 0.05$ (as in Maan & Cummings, 2012 for the eastern coachwhip). Finally, we used $\nu = 0.07$ as the Weber fraction of mammal photoreceptors (as in Chiao *et al*., 2000). To ensure that differences in Weber fraction estimates did not influence the results of this study, we also ran analyses using a single Weber fraction of $\nu = 0.10$ (results not shown). While the resulting contrast values differed between analyses depending on the Weber fraction, the relationships amongst contrast values did not differ.

With this information we calculated an overall color contrast, ΔS, for each predator. For tetrachromatic predators,

$$\Delta S = \{[(\omega_U\omega_S)^2(\Delta f_L - \Delta f_M)^2 + (\omega_U\omega_M)^2(\Delta f_L - \Delta f_S)^2 + (\omega_U\omega_L)^2(\Delta f_M - \Delta f_S)^2 + (\omega_S\omega_M)^2(\Delta f_L - \Delta f_U)^2 + (\omega_S\omega_L)^2(\Delta f_M - \Delta f_U)^2 + (\omega_M\omega_L)^2(\Delta f_S - \Delta f_U)^2]/[(\omega_U\omega_S\omega_M)^2 + (\omega_U\omega_S\omega_L)^2 + (\omega_U\omega_M\omega_L)^2 + (\omega_S\omega_M\omega_L)^2]\}^{1/2}$$

Trichromatic color contrasts are calculated as

$$\Delta S = \sqrt{\frac{\omega_S^2(\Delta f_L - \Delta f_M)^2 + \omega_M^2(\Delta f_L - \Delta f_S)^2 + \omega_L^2(\Delta f_S - \Delta f_M)^2}{(\omega_S\omega_M)^2 + (\omega_S\omega_L)^2 + (\omega_M\omega_L)^2}},$$

and dichromatic color contrasts are calculated as

$$\Delta S = \sqrt{[(\Delta f_L - \Delta f_S)^2/(\omega_S^2 + \omega_L^2)}.$$

In each equation above, the subscripts for f and ω refer to each photoreceptor present in that vertebrate class. Thus, tetrachromatic birds have four photoreceptors (U,S,M,L), trichromatic snakes three (S,M,L), and dichromatic mammals two (S,L). We calculated the brightness contrast, ΔL, as $|\Delta f_L/\omega_L|$, because the LWS cone is thought to control the achromatic perception of visual stimuli in many terrestrial animals (e.g. birds: Maier & Bowmaker, 1993; honeybees: Spaethe *et al*., 2001, Théry & Casas, 2002).

R code: This code is adapted for a tetrachromatic viewer. A simplification (using the above formulas as a guide) can be done to estimate contrasts for trichromatic or dichromatic viewers as well.

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Table AA1. Locations where salamanders were sampled for this project.

Location	Latitude	Longitude	Efts sampled	Erythristic *P. cinereus* sampled	Striped *P. cinereus* sampled
Cummington	42.45111111	-72.87555556	2	1	19
Lilly Pond	42.44027778	-72.82861111	15	20	20
Ashfield/Hawley	42.55388889	-72.87694444	1	7	20
Hawley	42.6	-72.86666667	9	15	20
Catamount	42.6475	-72.73916667	7	5	25
Savoy	42.57694444	-73.0675	2	18	20
Chesterfield	42.42083333	-72.88833333	15	1	20
Powell Brook	42.46138889	-72.95666667	3	4	20
Fisk Meadows	42.39222222	-72.81722222	15	20	20
Dubuque 1	42.54638889	-72.90472222	15	2	20
Dubuque 2	42.58222222	-72.91666667	7	7	20
Dubuque 3	42.55277778	-72.93972222	2	5	20
Sandisfield	42.14277778	-73.13	9	4	17
Cookson	42.06194444	-73.16	15	14	20

Table AA2. Co-occurrence of *N. viridescens* and erythristic *P. cinereus* across 40 sites in Western Massachusetts (striped *P. cinereus* were present at all 40 sites).

	N. viridescens present	*N. viridescens* absent
Erythristic *P. cinereus* present	14	0
Erythristic *P. cinereus* absent	18	8

CHAPTER III

BATESIAN MIMICS INFLUENCE THE EVOLUTION OF CONSPICUOUSNESS IN AN APOSEMATIC SALAMANDER

A paper to be submitted to a peer-reviewed journal

Abstract

Conspicuousness, or having high contrast to surrounding background, is a common feature of unpalatable species. Conspicuousness is thought to evolve to reduce confusion with palatable species (potential Batesian mimics) and this hypothesis hinges on the 'cost of conspicuousness,' in which conspicuousness increases predation likelihood more in palatable species than unpalatable species. Under this scenario three patterns are expected: 1) a positive relationship between mimic and model conspicuousness, 2) greater model conspicuousness in the presence of mimics, and 3) a mismatch between model and mimic conspicuousness, with mimics appearing less conspicuous. We tested these predictions in the salamander mimicry system involving *Notophthalmus viridescens* (model) and *Plethodon cinereus* (mimic). All predictions were supported, indicating that selection for Batesian mimicry not only influences the evolution of mimics, but also the models they resemble. These findings suggest that mimicry plays a larger role in the evolution of model warning signals than is currently appreciated.

Introduction

Batesian mimicry is an interspecific relationship in which a palatable 'mimic' species closely resembles an unpalatable 'model' species. In this relationship models are often conspicuous, exhibiting phenotypes that distinctly contrast with surrounding environment

through traits such as behavior, auditory cues, or, most commonly, coloration (Ruxton et al. 2004). Because conspicuous organisms stand out against their background, they can incur costs that include increased attention from predators in addition to the cost of resources invested in conspicuousness. Taking these costs into account, it is unclear why conspicuousness should evolve (Ruxton et al. 2004). One explanation is that despite these costs, conspicuous coloration serves as an effective signal of unpalatability to potential predators (this relationship is termed 'aposematism'; e.g. Maan and Cummings 2012). Conspicuousness is hypothesized to evolve in aposematic species to quickly train potential predators (Gittleman and Harvey 1980; Roper and Redston 1987; Lindström et al. 2001) and act as a mnemonic device for communicating unpalatability to potential predators (Guilford 1986; Speed 2000; Prudic et al. 2007). Conspicuousness may also ensure that aposematic individuals are not mistaken for palatable species (Fisher 1930).

Another hypothesis suggests that mimicry may drive models to evolve phenotypes distinct from mimics ('chase-away selection;' e.g. Poulton, 1890; Edmunds, 1974; Sherratt and Franks 2005; Franks et al. 2009), and that conspicuousness may evolve because conspicuous warning signals are more costly for palatable mimics than unpalatable models, as a result of heightened attention from predators (Speed and Ruxton 2010). Thus, conspicuousness in aposematic species may evolve to prevent palatable species from evolving perfect mimicry. If this is the case, selection for mimicry should drive mimics to track the conspicuousness of models, while selection on models will drive them to appear distinct from mimics. Additionally, the predation cost of conspicuousness will bar mimics from matching models in degree of conspicuousness. From this hypothesis we expect three patterns to emerge: 1) Through selection for mimicry, mimic conspicuousness will follow

model conspicuousness, leading to a correlation between mimic and model conspicuousness among locations. 2) Selection on models will drive them to be most conspicuous in the presence of mimics to remain distinctive to potential predators. 3) Due to the cost of conspicuousness, mimics will be unable to match model conspicuousness. This link between model conspicuousness and mimicry has long been predicted, but is understudied in natural systems of mimicry, particularly from the perspective of potential predators.

In this study, we tested three predictions generated from the hypothesis that conspicuousness may evolve in aposematic species through selection from predators to appear distinct from Batesian mimics. First, we tested for a correlation between mimic and model conspicuousness among locations. Second, we identified whether aposematic models were more conspicuous when syntopic (found together at the same locality) with mimics than when they were found alone. Third, we determined whether models are more conspicuous than their mimics. We evaluated these hypotheses in a mimicry system between two salamander species: *Notophthalmus viridescens* (the aposematic model) and its Batesian mimic, the erythristic color morph of *Plethodon cinereus* (see below). The accumulated evidence for mimicry in this system indicates that birds select for mimicry, specifically on the basis of coloration (Brodie and Brodie 1980; Tilley et al. 1982; Kraemer and Adams 2014). We used a bird visual model (*sensu* Kraemer and Adams 2014) to estimate color conspicuous of mimics and their aposematic models from the perspective of a relevant predator class, the tetrachromatic bird.

Materials and Methods

Study System

We examined hypotheses of conspicuousness in two salamander species: *Notophthalmus viridescens* (the aposematic model) and its Batesian mimic, the erythristic color morph of *Plethodon cinereus* (which is solid red-orange in coloration). These species are found in northeastern North America, and overlap at a broad regional scale. In western Massachusetts, *N. viridescens* can be found at a large number of locations while erythristic *P. cinereus* are present at a subset of these locations (Kraemer and Adams 2014). *Notophthalmus viridescens* has a triphasic life cycle that includes a secondary, juvenile (eft) stage, in which efts are terrestrial, vibrantly red-orange in coloration (Fig. 1), and contain tetrodotoxin, a neurotoxin, which makes them unpalatable to predators (Brodie 1968). Both larval and adult *N. viridescens* are aquatic and drab in coloration. *Plethodon cinereus* exhibits several discrete color morphs (including striped, unstriped, and erythristic), only one of which (the erythristic form) is qualitatively similar in coloration to efts (Fig. 1; Petranka 1998). Unlike *N. viridescens*, *P. cinereus* is palatable to predators and lacks tetrodotoxin (Brodie and Brodie 1980; Tilley et al. 1982). Of several potential vertebrate predators, birds are likely the predator class driving the evolution of mimicry in this system (Lotter and Scott 1977; Brodie and Brodie 1980; Tilley et al. 1982). Consistent with the hypothesis of mimicry, potential avian predators are able to discriminate between the coloration of non-mimic *P. cinereus* and efts, but not between the coloration of erythristic *P. cinereus* and efts (Kraemer and Adams 2014). Lending further support to this hypothesis, two empirical field studies have found that wild (Brodie and Brodie 1980) and captive (Tilley et al. 1982) bird

predators avoid *N. viridescens* and erythristic *P. cinereus* similarly, while they readily consume striped *P. cinereus*.

Salamander Collection and Color Quantification

In summer 2011, we collected 123 erythristic *P. cinereus* and 318 eft-stage *N. viridescens* salamanders from 32 locations in western Massachusetts, USA. While *N. viridescens* was present at all 32 locations, erythristic *P. cinereus* was found in a subset of the locations (supplemental material). All individuals were first anesthetized using tricaine methanesulfonate (MS-222: *P. cinereus*) or by applying benzocaine to the head

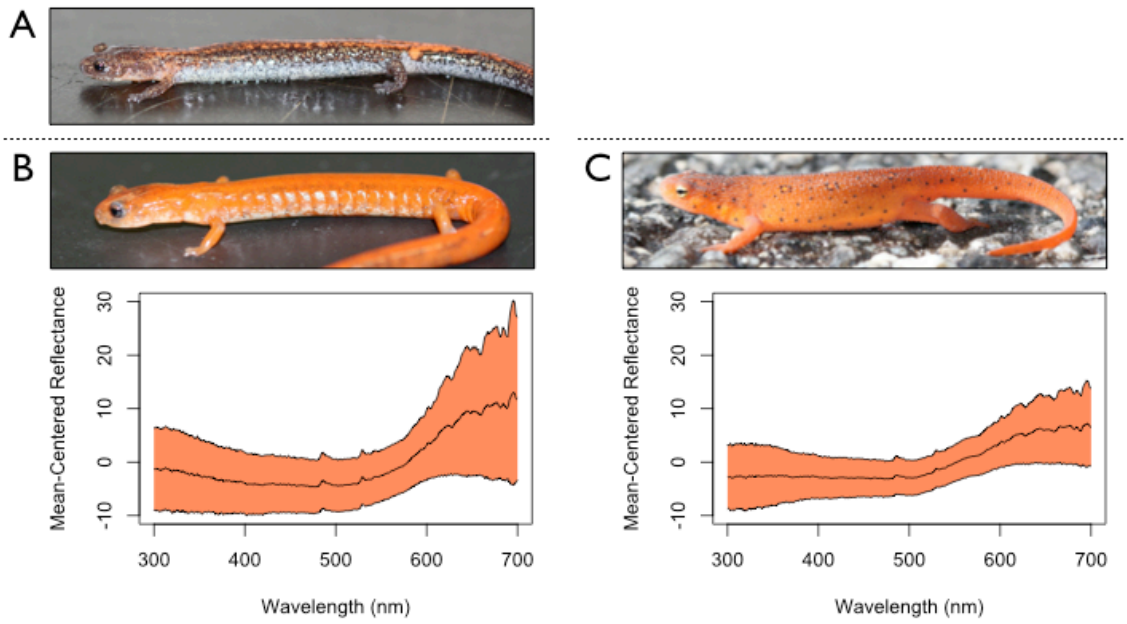

Figure 1. Representative salamanders from the *N. viridescens – P. cinereus* mimicry system. (A) non-mimic *P. cinereus*. (B) mimic (erythristic) *P. cinereus*. (C) an eft form of the model species *N. viridescens.* Spectrum height can be used to describe the brightness, while spectrum curvature is related to color. This study was focused on salamander coloration; thus, brightness-standardized reflectance spectra and standard deviations are included below salamanders used in the study.

(*N. viridescens*). There is the potential for differences in anesthetization to affect estimates of coloration between salamander groups. However, such physiologically-based color changes have only been observed in members of an unrelated salamander lineage (Garcia et al. 2003), and we did not observe such changes in our salamanders.

After anesthetization we measured spectral reflectance from a single point in the mid-dorsal region of each salamander using a portable JAZ-PX spectrometer (OceanOptics, Dunedin, FL) fitted with a 100 μm entrance slit, a pulsed xenon lamp, and a QR400-7-UV-BX reflectance probe. This probe was equipped with a tip that standardized the measured patch to a 2 mm diameter circle at a distance of 20 mm between probe and measured patch while excluding ambient light. We held the probe perpendicular to measured patches and used a Spectralon white reflectance standard between each animal to correct for drift in lamp intensity (see e.g., Kraemer et al. 2012). Collecting reflectance measurements at a perpendicular angle can introduce undesirable spectral glare, in particular under the circumstances in which coloration is influenced by structural elements in the target animal (Endler 1990). However, the dorsal coloration of these salamanders is composed of pigments with limited structural elements (Bagnara and Taylor 1970), which reduced the potential for problems associated with spectral glare in this study. We measured each spectrum at 1 nm intervals from 300 to 700 nm (see Fig. 1 for a visualization of spectral reflectance curves used in this study).

Terrestrial salamanders are most commonly found in soils with a thick layer of humus and decaying leaf matter (Grover 1998; Renaldo et al. 2011). Additionally, they are most active under cool and wet conditions (Grover 1998; Petranka 1998). Therefore, we obtained 10 reflectance spectra of naturally wet leaf litter on 9 June 2012 (from one location where

salamanders were collected: Lilly Pond Wildlife Management Area, Goshen, Massachusetts) to represent a typical background that predators would likely view salamanders against. Our sampling of background substrates is limited. However, all locations consisted of a similar complement of hemlock-northern hardwood forest, and thus we treated the backgrounds against which salamanders were viewed among locations as varying little.

Visual Model

We used a visual model developed by Vorobyev et al (2001) and modified to estimate salamander color conspicuousness (Kraemer and Adams 2014). This analytical model estimates discriminability of visual signals from the signal to noise ratio of predator photoreceptors (Vorobyev et al. 1998). The model yields estimates of visual contrast between targets and backgrounds (as in Maan and Cummings 2012). Because erythristic *P. cinereus* are thought to mimic *N. viridescens* on the basis of color, and not brightness (Kraemer and Adams 2014), selection on model phenotype to appear distinct from mimics will likewise be restricted to coloration. Thus, we used the visual model to calculate contrasts for the chromatic visual channel (ΔS), which summarizes the aspects of visual stimuli pertaining to coloration (i.e. chroma and hue). Large contrast values indicate similarly large and easily discriminable differences between targets and backgrounds from the predator's perspective, while smaller contrasts indicate similar coloration that are potentially indistinguishable. Specifically, contrasts greater than 1.0 are considered discriminable and potentially apparent to potential predators (Vorobyev et al. 1998)

The visual model requires reflectance measures of target (i.e. salamander) and background (i.e. leaf litter), background habitat irradiance (i.e. light environment), and

photoreceptor sensitivities of the predator. We used a forest shade irradiance measure reported elsewhere (*sensu* Kraemer and Adams 2014). Although there are several likely predators of salamanders in this system, prior work indicated that the evolution of mimicry in *P. cinereus* is likely the result of predation from tetrachromatic birds (Brodie and Brodie 1980; Tilley et al. 1982; Kraemer and Adams 2014). While we were unable to estimate the spectral sensitivities of particular bird predator species for this system (e.g. blue jay, hermit thrush, or robin), we were able to use known spectral sensitivities a related bird species (blue tit, Hart et al. 2000). Bird predators of these salamanders are tetrachromatic and likely have similar visual capabilities to the blue tit used to generate the visual model (Howard and Brodie 1973; Chen and Goldsmith 1986). A full description of visual model calculations and corresponding code are found in Kraemer and Adams (2014). All analyses were conducted in R 3.0.0 (R Development Core Team 2013).

Statistical Analyses

We used the following procedures to test for a relationship between mimicry and model conspicuousness:

Prediction 1: Model-Mimic Color Contrast Co-Variation

We performed a linear regression of erythristic *P. cinereus* and *N. viridescens* color contrasts across geographic locations to test the prediction that mimic coloration covaried with model coloration. In this analysis we used the average contrasts at each of 14 locations where erythristic *P. cinereus* and *N. viridescens* co-occurred.

Prediction 2: Relationship Between Model Color Conspicuousness and Mimicry

We performed a nested analysis of variance (ANOVA) using model color conspicuousness as the dependent variable, mimic presence/absence as the main group factor, and location as the sub-group factor to test the prediction that models will be more conspicuous in the presence of mimics.

Prediction 3: Relative Conspicuousness of Models and Mimics

We performed a nested ANOVA using color conspicuousness as the dependent variable, species as the main group factor, and location as the sub-group factor to test whether *N. viridescens* were more conspicuous than erythristic *P. cinereus*.

To assess the effect of differences in backgrounds on salamander conspicuousness, we performed two sensitivity analyses. First, we used each measured background spectrum as the background that salamander conspicuousness was estimated against and determined how this affected the resulting patterns of salamander conspicuousness. Second, we generated novel backgrounds using the average background spectrum and standard deviation of background spectra to evaluate if novel background spectra altered our observed patterns of salamander conspicuousness.

Results

Prediction 1: Model-Mimic Color Contrast Co-Variation

We found a significant and positive relationship between erythristic *P. cinereus* and *N. viridescens* color conspicuousness among the 14 locations where they were syntopic (R^2 = 0.357; P = 0.014; Fig. 2, inset). This relationship is stronger when the data is culled to include only locations represented by at least four *N. viridescens* and four erythristic *P. cinereus* individuals (R^2 = 0.624; P = 0.021).

Prediction 2: Model Color Variation and Mimic Presence

Using nested ANOVA, we found *N. viridescens* was more conspicuous to predators at locations where erythristic *P. cinereus* were present than at locations where erythristic *P. cinereus* were absent (df = 1; F = 6.54; P = 0.011; Fig. 2).

Prediction 3: Relative Conspicuousness of Models and Mimics

Using nested ANOVA, we found *N. viridescens* was significantly more conspicuous to predators than erythristic *P. cinereus* (mean *N. viridescens* ΔS = 3.13; mean erythristic *P. cinereus* ΔS = 1.11; df = 1; F = 164.19; P < 0.001; Fig. 2).

Further, the patterns we observed remained consistent when evaluated using different backgrounds. Thus, the results obtained here appeared robust to variation in backgrounds that salamanders are viewed against.

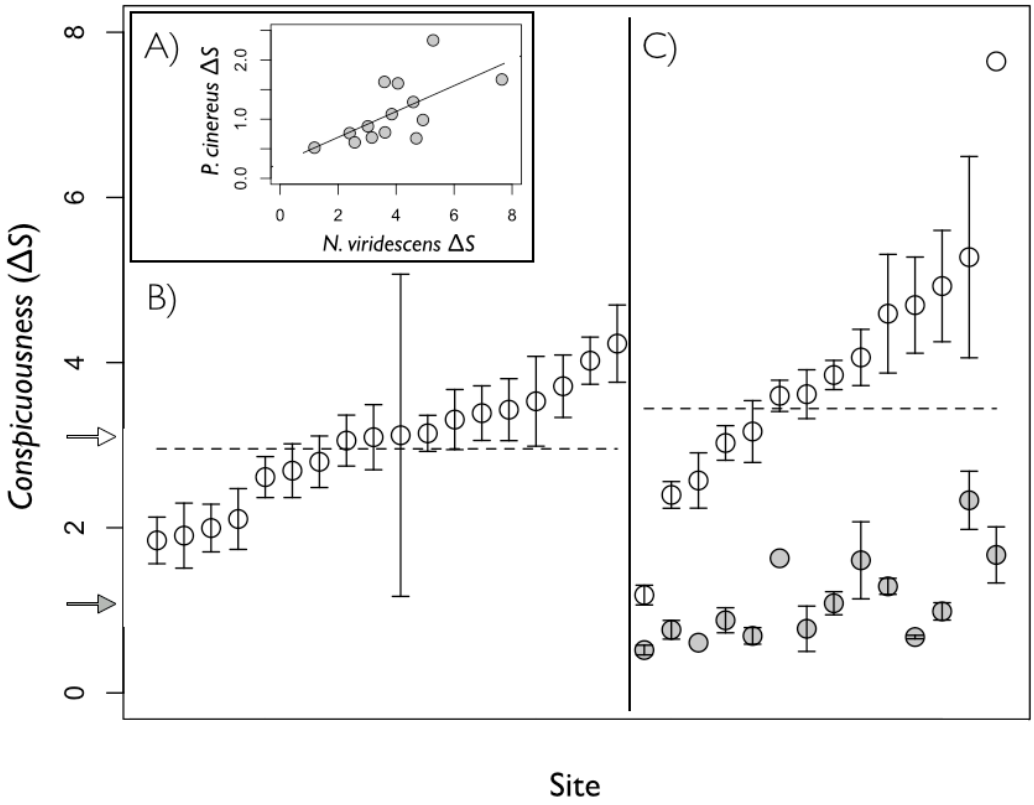

Figure 2. Color conspicuousness (Δ*S*) for *N. viridescens* and *P. cinereus* across locations. A) The inset illustrates the correlation between *N. viridescens* and *P. cinereus* conspicuousness. B) Estimates of conspicuousness for *N. viridescens* at locations without erythristic *P. cinereus*. C) Estimates of conspicuousness for *N. viridescens* (white points) and erythristic *P. cinereus* (grey points) at locations where both are found. The horizontal dotted lines illustrate how *N. viridescens* conspicuousness differs between locations with and without erythristic *P. cinereus*. Arrows denote mean conspicuousness scores for *N. viridescens* (white) and *P. cinereus* (grey). Standard error bars are included to show variation in the data.

Discussion

Aposematic species are often conspicuous, even though conspicuousness attracts the attention of potential predators. Fisher (1930), and more recently, Franks et al. (2009) hypothesized that conspicuousness may evolve in unpalatable species in response to mimicry, and we identify empirical evidence supporting this hypothesis in a salamander system. We found a significant, positive relationship among locations between the color conspicuousness of erythristic *P. cinereus* (the Batesian mimic) and *N. viridescens* (the

aposematic model species). Models were more conspicuous in the presence of mimics and were significantly more conspicuous than mimics at all locations.

Our observed relationship between mimic and model conspicuousness indicates that selection for mimicry drives mimics to resemble local models. This pattern is not unusual and has been observed in several taxa including frogs (Darst and Cummings 2006) and snakes (Greene and McDiarmid 1981). In these cases, as in our study, the adaptation of mimics to models is highly localized, with locations harboring different model and mimic phenotypes sometimes separated by only a few miles. For example, the tropical coral snake system reviewed by Greene and McDiarmid (1981) contains at least six discrete warning patterns in Central American coral snakes, each locally complemented by a nearly identical harmless snake mimic. These repeated patterns suggest that selection for mimicry can strongly influence the evolution of mimic phenotype.

We observed that models were significantly more conspicuous at locations where they were syntopic with mimics. This is an interesting finding that suggests mimicry may force models to evolve conspicuous warning signals to ‘avoid’ confusion with mimics (Fisher 1930; Franks and Noble 2004; Franks et al. 2009). Such a scenario, where mimics evolve toward models while models evolve away from mimics, is difficult to measure because of the highly multidimensional nature of warning signal design. We quantified this pattern in a natural mimicry system by examining the univariate measure of color conspicuousness, which forms an important part of a larger warning signal design that likely includes other dimensions (including color patterning, organism shape, and behavior). There is the potential for such an evolutionary ‘chase’ between models and mimics to extend to these phenotypic and behavioral dimensions as well. For example, aposematic species are

often described as bold and more lethargic or slow moving when compared to palatable relatives. Much like other aposematic species, but unlike other salamander species, *N. viridescens* efts are bold and can be found slowly traversing the forest floor in broad daylight. Such behaviors and other traits may evolve in concert with coloration as part of the 'aposematic syndrome' (Santos and Cannatella 2011) to ensure the distinctness of aposematic individuals to potential predators.

We also found that mimics were always less conspicuous than their models despite a location-level relationship between mimic and model conspicuousness. In most cases, the difference in conspicuousness between models and mimics was greater than one contrast unit (ΔS), which is typically considered to be the limit of discriminability for potential viewers (Vorobyev et al. 1998), indicating that these differences may be apparent to predators. This result is consistent with the hypothesis that there is a 'cost of conspicuousness' for Batesian mimics (Speed et al. 2010). Close resemblance to models may require mimics to become more conspicuous; however, conspicuousness can lead to increased attention from predators (Speed and Ruxton 2010). Conspicuousness is likely more costly for mimics because they do not possess the secondary defenses (e.g. unpalatability) that models carry. Consequently, selection to resemble models is countered by selection to avoid attention from predators, resulting in mimics that, though resembling their models, are less conspicuous. Although our results are concordant with this hypothesis, research that directly maps the adaptive landscape of model resemblance and conspicuousness in natural populations is needed to empirically test this hypothesis. Additionally, selection on mimics is influenced by the relative rate of encounters between predators, models, and mimics (reviewed in Ruxton et al. 2004). The adaptive landscape of mimicry will, therefore, depend critically on community

structure as well as morphological characteristics of the species involved. Alternatively, geographic variation in background or light environment could play a role in influencing the evolution of conspicuousness in this system. However, differences in the selective regimes imposed on models and mimics suggest that background and light environment variation is not sufficient to explain the patterns we observed.

The 'cost of conspicuousness' for Batesian mimics should create regions of phenotype-space where mimicry incurs an especially high fitness cost due to increased attention from predators (Franks et al. 2009). Under these circumstances, aposematic model species are predicted to evolve higher levels of conspicuousness when they are mimicked (Franks et al. 2009). We observed this pattern in a mimicry system between two salamander species. It would be valuable to assess whether this pattern is general across Batesian mimicry systems and at larger phylogenetic scales (e.g. are aposematic species that are mimicked generally more conspicuous than aposematic species without Batesian mimics?). If so, Batesian mimicry may influence macroevolutionary patterns in aposematic lineages, perhaps explaining why some aposematic lineages of poison frogs are more conspicuous than other lineages. Such a link between community ecology and macroevolutionary patterns of mimicry may, therefore, shed light on the evolutionary dynamics that direct the maintenance warning signals.

Conspicuousness in aposematic species may evolve for several reasons. Most commonly, conspicuousness is thought to evolve to improve predator recognition and/or memory of unpalatable species (e.g. Guilford 1986; Roper and Redston 1987). However, the relationship between defended species and other potential prey is increasingly considered as influencing the evolution of conspicuousness (e.g. Sherratt and Franks 2005; Franks et al.

2009). Here, we find evidence supporting the hypothesis that Batesian mimicry can drive the evolution of conspicuousness in an aposematic model species. The next step will be to identify the generality of this pattern and evaluate the impact of mimicry on the evolution of conspicuousness in aposematic species relative to other predator-driven forces (e.g. predator training and memory). A synthesis of these hypotheses will elucidate our understanding of how aposematic species evolve and why conspicuousness is a near-universal feature of aposematism.

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Appendix B

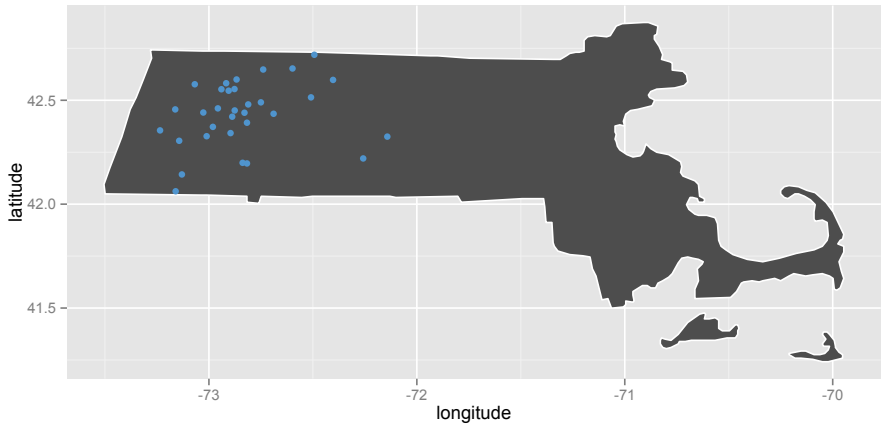
Table AB. Locations where salamanders were located and their respective sample sizes.

Location	Latitude	Longitude	*N. viridescens* sampled	Erythristic *P. cinereus* sampled
Cummington	42.451	-72.876	2	1
Lilly Pond	42.440	-72.829	15	20
Ashfield/Hawley	42.554	-72.877	1	7
Hawley	42.600	-72.867	9	15
Catamount	42.648	-72.739	7	5
Savoy	42.577	-73.068	2	18
Chesterfield	42.421	-72.888	15	1
Powell Brook	42.461	-72.957	3	4
Fisk Meadows	42.392	-72.817	15	20
Dubuque 1	42.546	-72.905	15	2
Dubuque 2	42.582	-72.917	7	7
Dubuque 3	42.553	-72.940	2	5
Sandisfield	42.143	-73.130	9	4
Cookson	42.062	-73.160	15	14
Peru	42.441	-73.027	15	0
Poland Brook	42.490	-72.750	7	0
Hiram H. Fox	42.342	-72.896	15	0
Montague	42.514	-72.509	2	0
Leyden	42.653	-72.598	13	0

Table AB *(continued)*.

Whatley	42.435	-72.689	15	0
Babbit Gribbons	42.325	-72.142	8	0
Palmer	42.220	-72.258	10	0
Westfield Watershed	42.196	-72.817	8	0
Tekoa Mtn	42.199	-72.838	15	0
Walnut Hill	42.327	-73.011	10	0
Fox Den	42.372	-72.981	13	0
Day Mountain	42.456	-73.162	3	0
Ashfield WCE	42.480	-72.811	16	0
Satans Kingdom	42.719	-72.493	12	0
Millers River	42.598	-72.403	11	0
Becket Mountain	42.305	-73.143	13	0
October Mountain	42.355	-73.235	15	0

Figure AB. Geographic positions of all sample locations.



CHAPTER IV

MIMICRY AND THE IMPORTANCE OF MODEL TOXICITY

A paper to be submitted to a peer-reviewed journal

Abstract

The resemblance between palatable mimics and unpalatable models in Batesian mimicry systems is tempered by many factors, including the toxicity of the model species. Model toxicity is thought to influence both the occurrence of mimicry and the evolution of mimetic phenotypes, and mimicry is most likely to persist when models are particularly toxic. Additionally, model toxicity may influence the evolution of mimetic phenotype in one of two ways. First, model toxicity may drive the evolution of mimetic fidelity such that mimicry will be most accurate when models are most toxic. Alternatively, model toxicity may allow inaccurate mimicry to evolve through a mechanism termed 'relaxed selection.' We tested these hypotheses in a salamander mimicry system between the model *Notophthalmus viridescens* and the mimic *Plethodon cinereus*, in which *N. viridescens* toxicity takes the form of tetrodotoxin. Surprisingly, though we discover spatial variation in model toxicity, we find no support for the hypotheses that model toxicity influences either the occurrence of mimicry or the evolution of mimic phenotype. Instead, variation in predator communities among localities or modern changes in environmental conditions may contribute to the patchy occurrence of mimicry in *P. cinereus*. Finally, limitations of predator perception or variation in the rate of phenotypic evolution of models and mimics may account for the evolution of imperfect mimicry in this salamander species.

Introduction

Batesian mimicry, in which a palatable mimic species resembles an unpalatable model species (Bates, 1862), has long stimulated questions on the origins of interspecific resemblance. For example, how does model unpalatability influence mimic phenotype? Experimental studies have found that model unpalatability can promote the evolution and maintenance of mimicry (Goodale and Sneddon, 1977; Lindström *et al.,* 1997), though the relationship between unpalatability and mimicry remains understudied (Darst and Cummings, 2006). In many Batesian mimicry systems, unpalatability is the consequence of toxic chemicals produced by the model (Ruxton *et al*., 2004). The toxicity of models may influence both the occurrence of mimicry (Endler, 1991; Lindström *et al*., 1997) and the evolution of mimic phenotype (Duncan and Sheppard 1965; Goodale and Sneddon, 1977; Lindström *et al*., 1997). It has been hypothesized that mimicry evolves and is maintained where models are most toxic (Endler, 1991; Lindström *et al*., 1997). Once established in a community, model toxicity may then influence the evolution of mimic phenotype in one of two ways. The classic expectation under natural selection is that in the presence of highly toxic models, mimics will evolve toward an ever more perfect resemblance of model phenotype (Fisher, 1930; Nur, 1970).

In contrast to the classic expectation, mimics are frequently observed that closely, but not perfectly, resemble their models (Ruxton *et al.*, 2004). In such cases, model toxicity is thought to play a role by deterring predators from attacking individuals carrying the model phenotype, including mimics. Under the relaxed selection hypothesis, predators will increasingly avoid mimics as the penalty for mistakenly attacking models increases, particularly through increased toxicity (Schmidt, 1958; Duncan and Sheppard, 1965;

Sherratt, 2002; Penney *et al*., 2012; Kikuchi and Pfennig, 2013). Thus, selection against imperfect mimics will decrease as models become more toxic, through a process termed 'stimulus generalization' (Duncan and Sheppard, 1965; Darst *et al*., 2006). Support for the relaxed selection hypothesis would consequently appear as a positive relationship between model toxicity and imperfect mimicry, with imperfect mimics occurring with highly toxic models (Goodale and Sneddon, 1977; Lindström *et al*., 1997).

The salamanders *Notophthalmus viridescens* (model) and *Plethodon cinereus* (mimic) are an ideal system to study the relationship between model toxicity and mimicry. Juvenile *N. viridescens* are bright orange-red and confer toxicity through tetrodotoxin (TTX; Yamashita and Mebs, 2001). TTX is a potent neurotoxin that blocks the pore region of voltage-gated sodium channels (Narahashi *et al.*, 1967) and potential predators find noxious (Brodie, 1968). After tasting and rejecting an *N. viridescens* individual, predators tend to avoid any prey that phenotypically resemble *N. viridescens* (Brodie, 1968). As such, predators often generalize this rejection to other species of orange-red salamanders (Howard and Brodie, 1973), including the erythristic color morph of *P. cinereus* (Brodie and Brodie, 1980; Tilley *et al*., 1982). Thus, erythristic *P. cinereus* are hypothesized to be Batesian mimics of *N. viridescens* (Lotter and Scott, 1977).

In this study, we tested for a relationship between model toxicity and mimicry by sampling terrestrial salamander communities from 32 localities across western Massachusetts. From each community we collected erythristic *P. cinereus* and *N. viridescens* for color quantification, and from the *N. viridescens* individuals we quantified dermal concentrations of TTX to estimate toxicity. If model toxicity influences the distribution of mimics and imperfect mimicry as hypothesized above, we first predict geographic variability

in *N. viridescens* toxicity. Next, if model toxicity influences the distribution of mimicry, we predict that the variation in *N. viridescens* toxicity will be associated with erythristic *P. cinereus* presence and abundance. Finally, if model toxicity influences the evolution of mimic phenotype, we predict that variation in *N. viridescens* toxicity will be associated with variation in mimic phenotype.

Materials and Methods

Study System

We examined the relationship between model toxicity and Batesian mimicry with two salamander species: *Notophthalmus viridescens* (model) and its Batesian mimic, the erythristic color morph of *Plethodon cinereus*. These species are widely distributed in northeastern North America and overlap across much of their respective ranges. *N. viridescens* has a triphasic life cycle that includes a secondary, juvenile (eft) stage, in which efts are terrestrial and strikingly red-orange in coloration (Petranka, 1998). Eft skin contains TTX (Mebs *et al.*, 2010), which is a potent neurotoxin that makes them highly unpalatable to predators (Brodie, 1968). The orange coloration of efts is interpreted as a warning signal of toxicity to most natural predators (Brodie, 1968). The terrestrial salamander *Plethodon cinereus* exhibits several discrete color morphs, including striped, unstriped, and erythristic. Only the erythristic form of *P. cinereus* is qualitatively similar in coloration to efts (Lotter and Scott, 1977). *Plethodon cinereus* are unlike *N. viridescens* in that they lack TTX and are palatable to predators (Brodie and Brodie, 1980; Tilley *et al*., 1982). Of several potential predator classes, birds are considered the predators driving the evolution of mimicry in this system (Lotter and Scott, 1977; Brodie and Brodie, 1980; Tilley *et al*., 1982; Kraemer and

Adams, 2014). Interestingly, while both *N. viridescens* and *P. cinereus* are commonly encountered at many localities in western Massachusetts, the erythristic color morph of *P. cinereus* is uncommon and typically occurs only in a subset of localities (Tilley *et al*., 1982; Kraemer and Adams, 2014).

Salamander Collection

In May and June 2011, we collected a total of 123 erythristic *P. cinereus* and 318 eft-stage *N. viridescens.* salamanders from 40 localities in western Massachusetts, USA. We visited each locality on three separate occasions, intensely searching under cover objects and in leaf litter for one hour per occasion. *N. viridescens* were found at 32 localities, and erythristic *P. cinereus* were found at 14 localities (Table 1). All individuals were first anesthetized using tricaine methanesulfonate (MS-222: *P. cinereus*) or by applying benzocaine to the head (*N. viridescens*). There is the potential for differences in anesthetization to affect estimates of coloration between salamander groups. However, such physiologically-based color changes have only been observed in members of an unrelated salamander lineage (Garcia *et al*., 2003), and we did not observe such changes in our salamanders.

Table 1. Sample localities and observed salamanders at each locality. Note that at the Chesterfield locality we observed hundreds of active *N. viridescens* at every sampling and were thus unable to estimate total observed *N. viridescens*.

Location	Latitude	Longitude	Total *N. viridescens*	Erythristic *P. cinereus*	Total *P. cinereus*
Cummington	42.451	-72.876	2	1	48
Lily Pond	42.440	-72.829	15	20	97
Ashfield/Hawley	42.554	-72.877	1	7	39
Hawley	42.600	-72.867	9	15	78
Catamount	42.648	-72.739	7	5	40
Savoy	42.577	-73.068	2	18	74
Chesterfield	42.421	-72.888	*	1	60
Powell Brook	42.461	-72.957	3	4	46
Fisk Meadows	42.392	-72.817	15	20	72
Dubuque 1	42.546	-72.905	15	2	61
Dubuque 2	42.582	-72.917	7	7	97
Dubuque 3	42.553	-72.940	2	5	66
Sandisfield	42.143	-73.130	9	4	21
Cookson	42.062	-73.160	15	14	76
Peru	42.441	-73.027	15	0	34
Poland Brook	42.490	-72.750	7	0	34
Hiram H. Fox	42.342	-72.896	15	0	72
Montague	42.514	-72.509	2	0	36
Leyden	42.653	-72.598	13	0	44

Table 1 (continued).

Whatley	42.435	-72.689	15	0	34
Babbit Gribbons	42.325	-72.142	8	0	15
Palmer	42.220	-72.258	10	0	22
Westfield Watershed	42.196	-72.817	8	0	75
Tekoa Mtn	42.199	-72.838	15	0	25
Walnut Hill	42.327	-73.011	10	0	50
Fox Den	42.372	-72.981	13	0	39
Day Mountain	42.456	-73.162	3	0	52
Ashfield WCE	42.480	-72.811	16	0	26
Satans Kingdom	42.719	-72.493	12	0	53
Millers River	42.598	-72.403	11	0	52
Becket Mountain	42.305	-73.143	13	0	68
October Mountain	42.355	-73.235	15	0	36

TTX Quantification

After collection, we field-preserved *N. viridescens* individuals in liquid nitrogen to be transported to Iowa State University (Ames, IA) for TTX quantification. From each individual, we removed a 5 mm diameter punch of skin (0.015 g) from the dorsal surface between the pelvic and pectoral girdle. Toxin extractions from each punch were prepared *sensu* Hanifin *et al*. (2002). Briefly, each sample was finely ground at room temperature with 600 µl of 0.1 M aqueous acetic acid and placed in a boiling water bath for five minutes, then centrifuged at 13000 RPM for 20 minutes. All resulting supernatant was transferred to a

Durapore PVDF 0.22 µm centrifugal filter tube (Millipore) and spun for 20 additional minutes at 13000 RPM. This extraction procedure is highly repeatable (r = 0.95; Hanifin *et al*., 1999), and thus does not introduce a significant degree of variation in toxicity estimates. We then quantified the concentration of TTX in each sample using Liquid Chromatography-Mass Spectrometry (LC-MS). To each sample, we added caffeine at a final concentration of 40 ng / µl and applied either 0.2 µl of sample to an LC-C18 column with 90% MeOH and 10% H_2O at a flow rate of 0.8 ml / min, or 1 µl of sample to an LC-C18 column with 100% MeOH at a flow rate of 0.8 ml / min. After separation, samples were sent to an Agilent QTOF 6540 mass spectrometer set to positive ion mode for detection and quantification. Concentration curves used to estimate sample TTX concentration were calculated from the known concentration of caffeine present in each sample (40 ng / µl) and TTX standards prepared from commercial TTX (Abcam). After estimating the concentration of TTX in each sample, we calculated the concentration of TTX in each gram of *N. viridescens* skin for analyses.

Color Quantification

After anesthetization in the field, we measured salamander spectral reflectance from a single point in the mid-dorsal region of each erythristic *P. cinereus* salamander using a portable JAZ-PX spectrometer (OceanOptics, Dunedin, FL) fitted with a 100 µm entrance slit, a pulsed xenon lamp, and a QR400-7-UV-BX reflectance probe. This probe was equipped with a tip that standardized the measured patch to a 2 mm diameter circle at a distance of 20 mm between probe and measured patch while excluding ambient light. We held the probe perpendicular to measured patches and used a Spectralon white reflectance

standard between each animal to correct for drift in lamp intensity (see e.g., Kraemer *et al*., 2012). Collecting reflectance measurements at a perpendicular angle can introduce undesirable spectral glare, in particular under the circumstances in which coloration is influenced by structural elements in the target animal (Endler, 1990). However, the dorsal coloration of these salamanders is composed of pigments with limited structural elements (Bagnara and Taylor, 1970), which reduced the potential for problems associated with spectral glare in this study. We measured each spectrum at 1 nm intervals from 300 to 700 nm.

Model/Mimic Matching

We used a visual model developed by Vorobyev *et al*. (2001) and modified to estimate salamander coloration (Kraemer and Adams, 2014) to estimate the degree of model-mimic matching at each locality. This analytical model estimates discriminability of visual signals from the signal to noise ratio of predator photoreceptors (Vorobyev *et al*., 1998). The model yields estimates of visual contrast between targets (model and mimic salamanders), and thus can be used to estimate similarity between mimics and models from the perspective of relevant predators. Because erythristic *P. cinereus* are thought to mimic *N. viridescens* on the basis of color, and not brightness (Kraemer and Adams, 2014), we used the visual model to calculate contrasts between models and mimics for the chromatic visual channel (ΔS), which describes the aspects of visual stimuli pertaining to coloration (i.e. chroma and hue). Large contrast scores indicate large and potentially discriminable differences between targets and backgrounds from the predator's perspective, while smaller contrasts indicate close mimicry that is potentially indistinguishable. Specifically, contrasts greater than 1.0 are

considered discriminable and potentially apparent to potential predators (Vorobyev *et al*., 1998).

The visual model requires reflectance measures of targets (i.e. models and mimics), background habitat irradiance (i.e. light environment), and photoreceptor sensitivities of the predator. We used a forest shade irradiance measure reported elsewhere (*sensu* Kraemer and Adams, 2014). Although there are several likely predators of salamanders in this system (Petranka, 1998), prior work indicated that the evolution of mimicry in *P. cinereus* is likely the result of predation from tetrachromatic birds (Brodie and Brodie, 1980; Tilley *et al*., 1982; Kraemer and Adams, 2014), which we approximated by using the spectral sensitivities of the blue tit (Hart *et al*., 2000). A full description of visual model calculations are found in Kraemer and Adams (2014). All analyses were conducted in R 3.0.2 (R Development Core Team, 2013).

Color Variation

We estimated variation in erythristic *P. cinereus* coloration by performing a principal components analysis on the salamander reflectance data (*sensu* Endler and Thèry, 1996; Grill and Rush, 2000; Rosenblum, 2006). In this representation, PC1 is interpreted as brightness, or the total amount of light reflected from each patch, while the remaining PC-axes (PC_2-PC_{401}) represent aspects of chroma and hue (Endler, 1990; Endler and Thèry, 1996; Grill and Rush, 2000) and thus the color that each spectrum represented. From these data we calculated the multivariate dispersion of salamander coloration at each locality (using ‘betadisper’ in the vegan library in R).

Statistical Analyses

We first tested for significant variation in *N. viridescens* toxicity across localities. We then tested for a relationship between model toxicity and mimicry by comparing the mean and variance of *N. viridescens* toxicity at each locality to the presence and abundance of mimics. Finally, we tested for a relationship between model toxicity and imperfect mimicry by comparing the mean and variance of *N. viridescens* toxicity at each locality to the degree of erythristic *P. cinereus – N. viridescens* color match and to the degree of multivariate dispersion of erythristic *P. cinereus* coloration at each locality.

TTX Variation

We performed an analysis of variance (ANOVA) with *N. viridescens* toxicity as estimated from LC-MS as the dependent variable and locality as the group factor to identify if there is significant variation in *N. viridescens* toxicity across localities.

Toxicity and Mimicry

To identify if mimics occurred where models were most toxic, we performed an ANOVA with mean *N. viridescens* toxicity as the dependent variable and presence of erythristic *P. cinereus* as the group factor. We then performed an ANOVA with the variance of *N. viridescens* toxicity as the dependent variable and presence of erythristic *P. cinereus* as the group factor. We conducted a series of linear regressions between the mean *N. viridescens* toxicity and erythristic *P. cinereus* abundance (as calculated by total number of mimics observed, the ratio of mimics to models, and the ratio of mimics to all *P. cinereus* observed at the locality). We also conducted a series of linear regressions between the

variance of *N. viridescens* toxicity and erythristic *P. cinereus* abundance to identify if any measure of mimic abundance co-varied with model toxicity.

Toxicity and Imperfect Mimicry

To test for a relationship between imperfect mimicry and model toxicity we performed two sets of linear regressions, 1) between *N. viridescens* toxicity and the degree of color match between *N. viridescens* and erythristic *P. cinereus* from the perspective of bird predators, and 2) between *N. viridescens* toxicity and the multivariate dispersion of erythristic *P. cinereus* coloration among localities. Each linear regression was performed using both mean *N. viridescens* toxicity and the variance in *N. viridescens* toxicity calculated at each locality.

Results

TTX Variation

We found significant variation in *N. viridescens* toxicity across localities (Figure 1; adjusted $R^2 = 0.013$; $F = 4.85$; $P = 0.028$), with individuals at some localities possessing no detectable TTX and individuals at other localities possessing over 1.0 mg TTX / g of *N. viridescens* skin, which is comparable to many but the most toxic populations of *Taricha* newts in the Pacific Northwest (Hanifin *et al.,* 1999). One specimen outlier, possessing over 5 mg TTX / g skin, was removed from analysis.

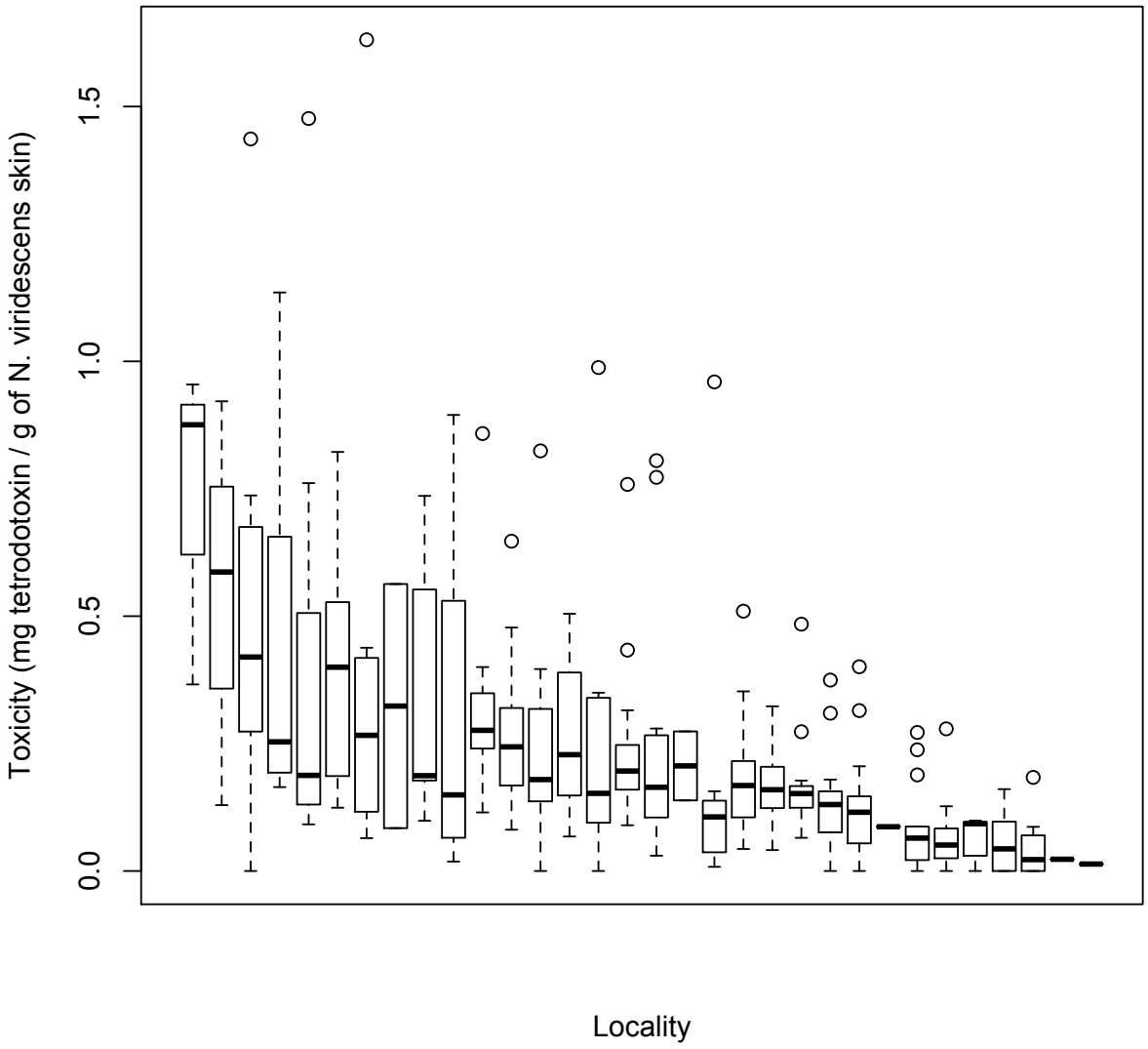


Figure 1. Variation in the concentration of tetrodotoxin of *N. viridescens* individuals among localities.

Toxicity and Mimicry

We observed no relationship between *N. viridescens* toxicity and erythristic *P. cinereus* presence (Table 2). We also found no relationship between *N. viridescens* toxicity and erythristic *P. cinereus* abundance as calculated by number of erythristic *P. cinereus* individuals observed at a locality, the ratio of observed erythristic *P. cinereus* to *N.*

viridescens, or the ratio of erythristic *P. cinereus* to total salamanders observed at a locality (Table 2).

Table 2. The relationship between *N. viridescens* (model) toxicity and mimicry. Analyses on the left summarize comparisons between mean model toxicity at each locality and mimicry, while analyses on the right correspond to comparisons between the variance of model toxicity at each locality and mimicry. A) Note no relationship between model toxicity and mimic presence or any measure of mimic abundance. B) Also note the lack of relationship between model toxicity and either measure of imperfect mimicry.

	Mean Toxicity			**Variance in Toxicity**		
A)	**Sum Sq**	**F value**	**P**	**Sum Sq**	**F value**	**P**
Erythristic *P. cinereus* presence	5247.000	0.180	0.674	1.463×10^9	0.303	0.586
Erythristic *P. cinereus* abundance	20.200	0.384	0.547	37.700	0.697	0.423
Erythristic *P. cinereus* : *N. viridescens*	1.580	0.193	0.669	1.580	0.193	0.669
Erythristic *P. cinereus* : total *P. cinereus*	0.002	0.214	0.652	0.003	0.405	0.539
B)	**Sum Sq**	**F value**	**P**	**Sum Sq**	**F value**	**P**
Model-mimic matching	0.025	0.099	0.759	0.023	0.077	0.787
Mimic multivariate dispersion	104.000	0.132	0.723	237.000	0.401	0.541

Toxicity and Imperfect Mimicry

We also found no relationship between *N. viridescens* toxicity and degree of color match between *N. viridescens* and erythristic *P. cinereus*, nor between *N. viridescens* toxicity and multivariate dispersion of erythristic *P. cinereus* coloration (Table 2). Thus, these results do not align with either hypothesized link between mimicry and model toxicity, predicts both a positive relationship between *N. viridescens* toxicity and the abundance of mimics and a positive relationship between *N. viridescens* toxicity and imperfect mimicry.

Discussion

Theoretical (Pilecki and O'Donald, 1971) and experimental (Duncan and Sheppard, 1965; Goodale and Sneddon, 1977; Lindström *et al*., 1997) studies have indicated that model unpalatability may influence the evolution and maintenance of Batesian mimics. The aim of this study was to observe the microevolutionary patterns that such a relationship predicts in a natural mimicry system of two salamander species. We found significant variation in model (*Nothophthalmus viridescens*) toxicity among localities. Despite this variation, toxicity was not linked to the presence or abundance of mimics (erythristic *Plethodon cinereus* salamanders). We also found no relationship between model toxicity and two measures of imperfect mimicry. As such, while we discovered considerable spatial variation in model toxicity, we found no support for the hypotheses that model toxicity influences either the maintenance of mimicry or the evolution of the mimetic phenotype.

Erythristic *P. cinereus* are rare mimics of *N. viridescens* and never exceed ~25% of the *P. cinereus* population at any locality in western Massachusetts (Tilley *et al*., 1982; Kraemer and Adams, 2014). For *N. viridescens* unpalatability to influence the distribution of

erythristic *P. cinereus* in western Massachusetts, we predicted significant variation in model toxicity among localities (Figure 1). Despite the variation we found, there was no relationship between model toxicity and the distribution of mimicry. Interestingly, we had previously identified a relationship between mimicry and model presence in this system (Kraemer and Adams, 2014). When viewed in light of this study, these results suggest that the presence of unpalatable models at a locality is necessary, but not sufficient, to maintain the presence of mimicry. Furthermore, variation in unpalatability does not further influence the maintenance of mimicry in this system. While the breakdown of mimicry in the absence of models is well established, such examples of mimicry breakdown typically involve species boundaries at a larger scale than examined here (e.g. Platt and Brower, 1968; Greene and McDiarmid, 1981; Pfennig *et al*., 2001; Prudic and Oliver, 2008). Little research exists to explain the absence of mimics at localities within the ranges of both models and mimics. Other factors, such as selection from mammal predators (Kraemer *et al*., in preparation), may limit the distribution mimics to a subset of localities where *N. viridescens* are present.

Examples of Batesian mimicry are often used to illustrate the power of natural selection to drive phenotypic convergence (Ruxton *et al*., 2004; Forbes, 2009), where selection for mimicry is predicted to result in mimics well matched to their model species (Ruxton *et al*., 2004). Despite this expectation, many species imperfectly resemble their models, such as hoverflies (Penney *et al*., 2012), snakes (Savage and Slowinski, 1992), and spiders (Edmunds, 2000). Imperfect mimicry may evolve under several scenarios (Kikuchi and Pfennig, 2013), including relaxed selection on mimics (Schmidt, 1958; Duncan and Sheppard, 1965; Sherratt, 2002; Penney *et al*., 2012). Relaxed selection states that as the cost:benefit ratio of attacking models and mimics increases, a smaller proportion of mimics

will be attacked. Thus, as models become increasingly unpalatable, selection on mimics will relax (see Figure 2 from Kikuchi and Pfennig, 2013), resulting in imperfect mimics that are as fit as perfect mimics. Consequently, where models are the most toxic we predict that mimic phenotype will be the most 'imperfect' and most variable. At the 14 localities where both erythristic *P. cinereus* and *N. viridescens* were present, we found no relationship between model toxicity and our two measures of mimetic imperfection. We also found no relationship between imperfect mimicry and either strict model abundance or relative abundance of mimics and models (Table 2). Alternatively, variation in mimic handling time or size among localities may contribute to the cost:benefit ratio of attacking models and mimics and therefore result in relaxed selection on mimics. However, we observed no differences in mimic behavior or size among localities (*Kraemer, pers. obs.*). Thus, the variation in imperfect mimicry we observed is unlikely to be due to relaxed selection on mimics.

Kikuchi and Pfennig (2013) summarize 11 non-exclusive mechanisms that may contribute to the evolution of imperfect mimicry, including relaxed selection. While we found no support for relaxed selection, five alternative mechanisms may play a role in the evolution of mimicry in *P. cinereus*. First, most color variation in erythristic *P. cinereus* appears to be below the level of detection for relevant bird predators (Kraemer and Adams, 2014), which may prevent predators from distinguishing between models and 'imperfect' mimics (the 'eye-of-the-beholder' hypothesis; Cuthill and Bennet, 1993; Dittrich *et al.*, 1993). Alternatively, differences in brightness (Kraemer and Adams, 2014), body shape, or behavior between erythristic *P. cinereus* and *N. viridescens* may allow for satyric mimicry in this system, which requires imperfect mimics to possess sensory cues that confuse potential

predators, resulting in greater latency between detection and attack (Howse and Allen, 1994). Patterns of conspicuousness between erythristic *P. cinereus* and *N. viridescens* suggest that chase-away selection, in which models evolve away from mimics and a time-lag prevents the immediate evolutionary response of mimics (Nur, 1970; McGuire *et al*., 2006; Franks *et al*., 2009), may be responsible for imperfect mimicry (Kraemer *et al.,* in preparation). Finally, multiple predators may select for imperfect mimicry in this system, with some predators selecting for mimicry while other predators impose opposing selective pressures on mimic phenotype. This possibility is particularly interesting, as birds are considered the only predators that select for mimicry in *P. cinereus* (Lotter and Scott, 1977; Brodie and Brodie, 1980; Tilley *et al*., 1982). Other predators, such as mammals, likely forage on *P. cinereus* (Petranka, 1998), but may not perceive erythristic *P. cinereus* as mimics of *N. viridescens* (Kraemer and Adams, 2014). Instead, mammals likely select for inconspicuousness and novel phenotypes in *P. cinereus* (Kraemer *et al.,* in preparation). It is unclear how these five mechanisms may interact to contribute to imperfect mimicry in *P. cinereus*. A better understanding of the predators involved in this system and the variation in predation pressure among localities may help to disentangle the influence of these mechanisms.

In conclusion, we find no influence of model toxicity on model presence, abundance, or degree of imperfection among localities. The absence of mimics where models are present is an interesting problem that has been given little attention. We find that in *P. cinereus*, the absence of the mimic morph at 18 localities where the model occurs cannot be attributable to variation in model toxicity. Instead, other locality-specific factors, such as variation in the predator community (Pekár *et al*., 2011) or availability of alternative prey (Carpenter and Ford, 1953), may influence the distribution of the mimic morph. Additionally, changes in

land use, climate, and predator communities among the sampled localities may contribute to the current distribution of erythristic *P. cinereus*. Work that examines the historical and current distribution of *P. cinereus* color morphs in light of such changes may be able to elucidate the impact of environmental change on the distribution of mimicry in this system. Our findings also allow us to reject the hypothesis that imperfect mimicry in *P. cinereus* is due to relaxed selection from predators. Instead, imperfect mimicry in *P. cinereus* may be due to limitations of predator vision, the ability of mimics to confuse predators, a time lag between model and mimic evolution, or the existence of multiple predators, each of which will need to be explicitly tested to better understand the distribution and evolution of mimicry in this salamander species.

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CHAPTER V

BOTH NOVELTY AND CONSPICUOUSNESS INFLUENCE SELECTION ON *PLETHODON CINEREUS* COLOR MORPHS BY MAMMAL PREDATORS

A paper to be submitted to a peer-reviewed journal

Abstract

Predators influence selection on prey coloration and patterning, yet how selection may differ among predators is seldom considered. In particular, prey color patterns that indicate unpalatability to some predator species may not carry the same signal for other predators. We test several hypotheses of selection on coloration and pattern between mammal predators and the polymorphic salamander *Plethodon cinereus*, which, under an avian visual system appears as the toxic newt *Notophthalmus viridescens*. We fit each hypothesis against field observations of mammalian predation on salamander clay replicas. We then develop a novel analytical procedure that enables the combination of multiple hypotheses in a likelihood framework. We find that mammals do not follow any individual hypothesis proposed, including the hypothesis of mimicry. Instead, mammals use visual cues while foraging by avoiding unfamiliar, novel prey, and attacking conspicuous prey. We hypothesize that mammals may help to maintain color polymorphism within populations of *P. cinereus* by avoiding novel, unfamiliar color morphs. Additionally, selective pressures from multiple predators and variation in predator communities among sites may contribute to the maintenance of color polymorphism within and among localities in *P. cinereus*.

Introduction

Predation exerts strong selective pressures on prey phenotype, often acting as a creative force driving the evolution of new phenotypic traits that deter predators (Endler, 1988). For example, defensive structures present in prey populations that are sympatric with predators are frequently lost in predator-poor environments (Bell *et al*., 1993; Reimchen, 2000), and experimental evidence has shown that the presence of predators exerts strong selective pressures on life history and other phenotypic traits (Reznick *et al.,* 1990; 1997). High rates of predation can drive prey species to mature at smaller sizes (Reznick and Endler, 1982) or to evolve extremely high toxicity (Brodie *et al.,* 2002). However, while such studies inform on how predation can act as a constructive force of selection that drives the evolution of new phenotypes, less attention has been given to how predation can maintain phenotypic diversity within prey species (Bond, 2007).

A common phenotypic trait to evolve in response to predation is coloration and patterning, which can directly influence predator detection and identification of potential prey (Endler, 1986). In many species, such selection from predators has resulted in color polymorphism, where multiple color morphs exist for a single prey species (reviewed in Bond, 2007). Though different predator behaviors may result prey color polymorphism, in many cases the evolution of color polymorphism is strongly directed by the visual capabilities of potential predators (Ruxton *et al*., 2004). Depending on the predator's perception of prey coloration during prey detection and classification, overall selection on different prey color morphs may fit several different hypotheses, which may or may not maintain color polymorphisms. First, predators may avoid prey color morphs that resemble

an unpalatable species, thus driving the evolution of Batesian mimics that closely resemble their unpalatable models (the Mimicry Hypothesis, Figure 1A; Bates, 1862).

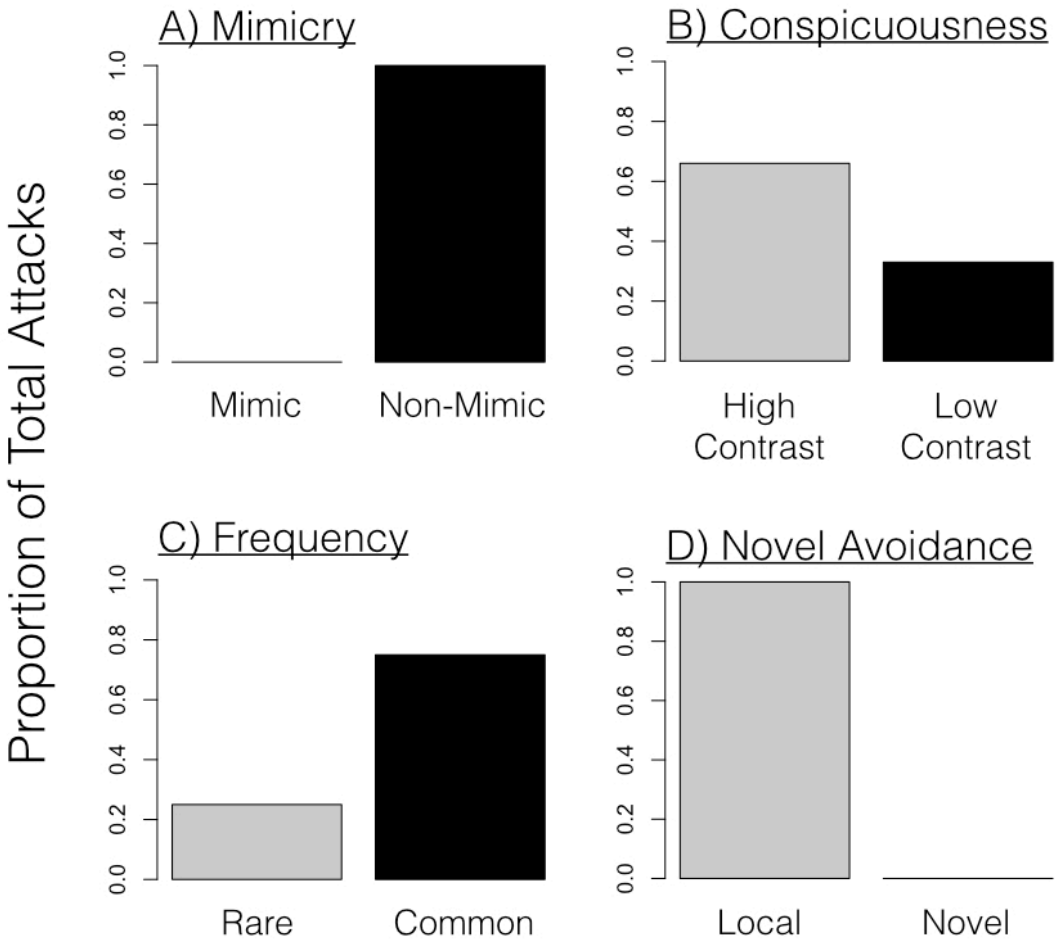

Figure 1. Schematic of predator attacks expected under each single hypothesis. A) The Mimicry Hypothesis states that mimics will be avoided, resulting in 100% of attacks on non-mimics. B) The Conspicuousness Hypothesis states that prey with the highest contrast with background will be attacked most. In this example, the gray morph is twice as conspicuous as the black morph, thus sustaining twice as many attacks. C) The Frequency Hypothesis states that rare morphs will be attacked less frequently and common morphs will be attacked more. In this example, the gray morph is rare (making up 25% of the local population and thus 25% of total attacks), while the black morph is more common (making up 75% of the local population and thus 75% of total attacks). D) The Novel Avoidance Hypothesis states that morphs not locally encountered are avoided. In this example, the gray morph is locally encountered by predators and thus attacked, while the black morph is absent and is thus avoided by predators. Note that the Null Hypothesis predicts no difference in attacks among morphs (not depicted), and the Combined Hypothesis has different predictions depending on the weights given for each single hypothesis (not depicted).

Alternatively, foraging predators may identify and target prey whose coloration has greater contrast with the background environment (i.e. more conspicuous), thereby driving the evolution of prey difficult for predators to initially detect (the Conspicuousness Hypothesis, Figure 1B; Endler, 1978). Predators may also recognize, and then attack, those prey that are most common in the environment, resulting in frequency-dependent selection against common prey (the Frequency Hypothesis, Figure 1C; Allen, 1988). On the other hand, predators may avoid unfamiliar prey (the Novel Avoidance Hypothesis; also called 'dietary conservatism', Figure 1D; Marples *et al*., 2007), or predators may not use coloration or patterning in any way during foraging, perhaps instead utilizing other senses, such as smell (the Null Hypothesis; Endler, 1986; Hughes *et al.*, 2010; Ruxton, 2009). Importantly, these hypotheses are not necessarily exclusive. Predators may incorporate several of the above strategies into a single composite behavior when responding to color cues in potential prey (the Combined Hypothesis; Endler, 1986). These hypotheses can be difficult to observe directly, and are best tested through the observation of predation events themselves (e.g. Kikuchi and Pfennig, 2010).

In salamanders, predation is a major source of mortality, and a diversity of coloration and pattern has consequently evolved in response to predators (Petranka, 1998). In some taxa, this includes color polymorphism, or multiple color morphs, within species. In the salamander *Plethodon cinereus*, three distinct color morphs are typically encountered, including the solid red-orange 'erythristic' morph, the solid black 'unstriped' morph, and the 'striped' morph that possesses a single red dorsal stripe on a dark background (Lotter and Scott, 1977; Figure 2A-C). Birds are hypothesized to associate erythristic *P. cinereus*, which

are palatable (Tilley *et al*., 1982), with similarly colored juveniles of the highly toxic newt *Notophthalmus viridescens*, making erythristic *P. cinereus* Batesian mimics of *N. viridescens* (Lotter and Scott, 1977; Brodie and Brodie, 1980; Tilley *et al.*, 1982). A recent study found that bird predators with tetrachromatic vision should be able to discriminate non-mimic *P. cinereus* from *N. viridescens*, but not mimic *P. cinereus* from *N. viridescens* on the

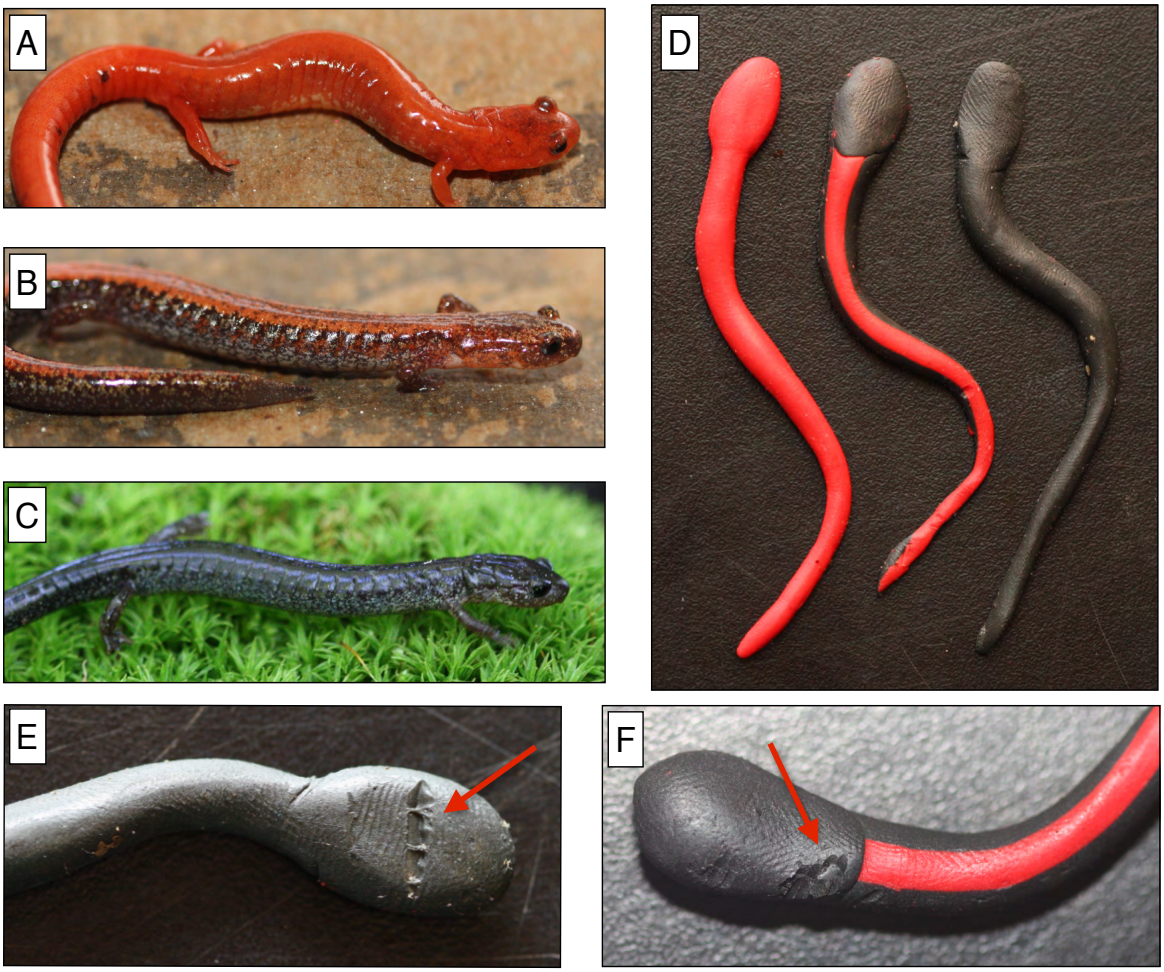

Figure 2. *Plethodon cinereus* color morphs, clay replicas, and typical mammalian impressions. A) An erythristic *P. cinereus* salamander; B) a striped *P. cinereus* salamander; C) an unstriped *P. cinereus* salamander. D) Representative clay replicas of each salamander morph: Left, erythristic; center, striped; right, unstriped. Typical mammalian impressions on an E) unstriped replica and an F) striped replica.

basis of coloration. Birds are thus capable of imposing selection on *P. cinereus* coloration consistent with Batesian mimicry (Kraemer and Adams, 2014). In contrast, Kraemer and Adams (2014) found that visual models for diurnal, dichromatic mammals are unable to discriminate salamanders on the basis of coloration, suggesting that visual constraints might prevent diurnal mammal predators from selecting for mimicry in this system. However, when mammalian predators use visual cues during hunting, they may utilize aspects distinct from coloration (such as brightness), thereby selecting for color patterning in a manner different from birds. For example, differences in patterning between *P. cinereus* color morphs may differentially influence how mammals detect or recognize salamanders as prey, consequently attacking one morph more frequently than other salamander morphs. In the present study, we test several hypotheses of selection on *P. cinereus* color pattern by mammal predators, specifically the Mimicry, Conspicuousness, Frequency, Novel Avoidance, and Null hypotheses as described above. We test the fit of each hypothesis against field observations of predation on clay replicas of each *P. cinereus* color morph. We then use a novel analytical procedure that enables the evaluation of a combination of multiple predation hypotheses in a likelihood framework.

Materials and Methods

Study System

The salamander species *Plethodon cinereus* is the most common vertebrate species in New England forests (Burton and Likens, 1962). Three discrete color morphs can be found (Figure 2A-C), but many populations are color dimorphic (Lotter and Scott, 1977). Based on color and brightness, predators are predicted to interact with each morph somewhat

differently (Lotter and Scott, 1977). In particular, the erythristic color morph is thought to be a Batesian mimic of the toxic salamander *Notophthalmus viridescens* (Brodie and Brodie, 1980) due to similarity in coloration (Kraemer and Adams, 2014). Avian predators are thought to drive the evolution of color mimicry in this system (Brodie and Brodie, 1980; Tilley *et al*., 1982; Kraemer and Adams, 2014), while the impacts of additional predators, such as mammals and snakes, are less understood. In North America, mammals are an important salamander predator (Petranka, 1998). Mammalian attacks on salamanders are less well documented as compared to attacks from snakes and birds (e.g. Lotter and Scott, 1977, Arnold, 1982; Fenster and Fenster, 1996; Adams, 1999), and likely are due to opossums, raccoons, skunks, opportunistic rodents, and other small carnivorous mammals (Stebbins, 1954; Huheey and Stupka, 1967; Brodie *et al.,* 1979; Beachy, 1991; Dodd, 1991; Petranka, 1998). These dichromatic mammalian species have visual capabilities far different from bird predators, particularly through a narrower range of visual sensitivities (Chen and Goldsmith, 1986; Jacobs, 1993; Kraemer and Adams, 2014). While mammals are known to use non-visual senses while foraging (e.g., smell: Pyare and Longland, 2001; Hughes *et al*., 2010; but see Discussion), they also utilize visual cues during the predation process. As such, it is of interest to determine whether mammalian predators differentially attack *P. cinereus* color morphs in accordance with several hypotheses in which predator behavior is mediated by visual cues (see *Expected Attacks For Each Hypothesis* below). We tested predictions in the field using clay replicas of salamander morphs that were exposed to mammalian predators for four to five days. At the conclusion of this period, we collected the replicas, scored them for mammalian attacks, and compared observed attack rates to attack rates expected under each hypothesis (described below).

Replica Construction

We constructed clay replicas out of Sculpey III® polymer clay. This clay does not harden under field conditions but retains impressions made by potential predators, thus serving as a record of predation events over several days (e.g. Brodie, 1993; Pfennig *et al*., 2001; Kuchta, 2005). We formed models to resemble *P. cinereus* morphs that were present at the four localities (striped, unstriped, and erythristic; Figure 2A-C). To construct the replicas, we hand-shaped 1.5 grams of black or red clay to resemble a basic salamander form with a head and tapered body (Figure 2D). We made 300 replicas of each salamander color morph, for a total of 900 clay replicas.

Replica Distribution and Retrieval

We chose four locations in western Massachusetts based on the presence and frequency of *P. cinereus* color morphs. At two locations striped and erythristic *P. cinereus* are present (Fisk and Lily; located in Fisk Meadows Wildlife Management Area and Lily Pond Wildlife Management Area, respectively), while at the other two locations striped and unstriped *P. cinereus* are frequently encountered (Coys and Palmer; located in Coys Hill Wildlife Management Area and Palmer Wildlife Management Area, respectively; Figure 3A). We placed the replicas in the field from 28 May to 31 May 2012, and we collected the replicas from 1 June to 5 June 2012 such that each replica was exposed to predators for four to five days.

We distributed an equal proportion of each color morph at all four localities, so any deviation from equal predation across morphs strongly suggests that predators are using coloration to identify potential prey. We placed 15 replicas (five of each morph) at every five

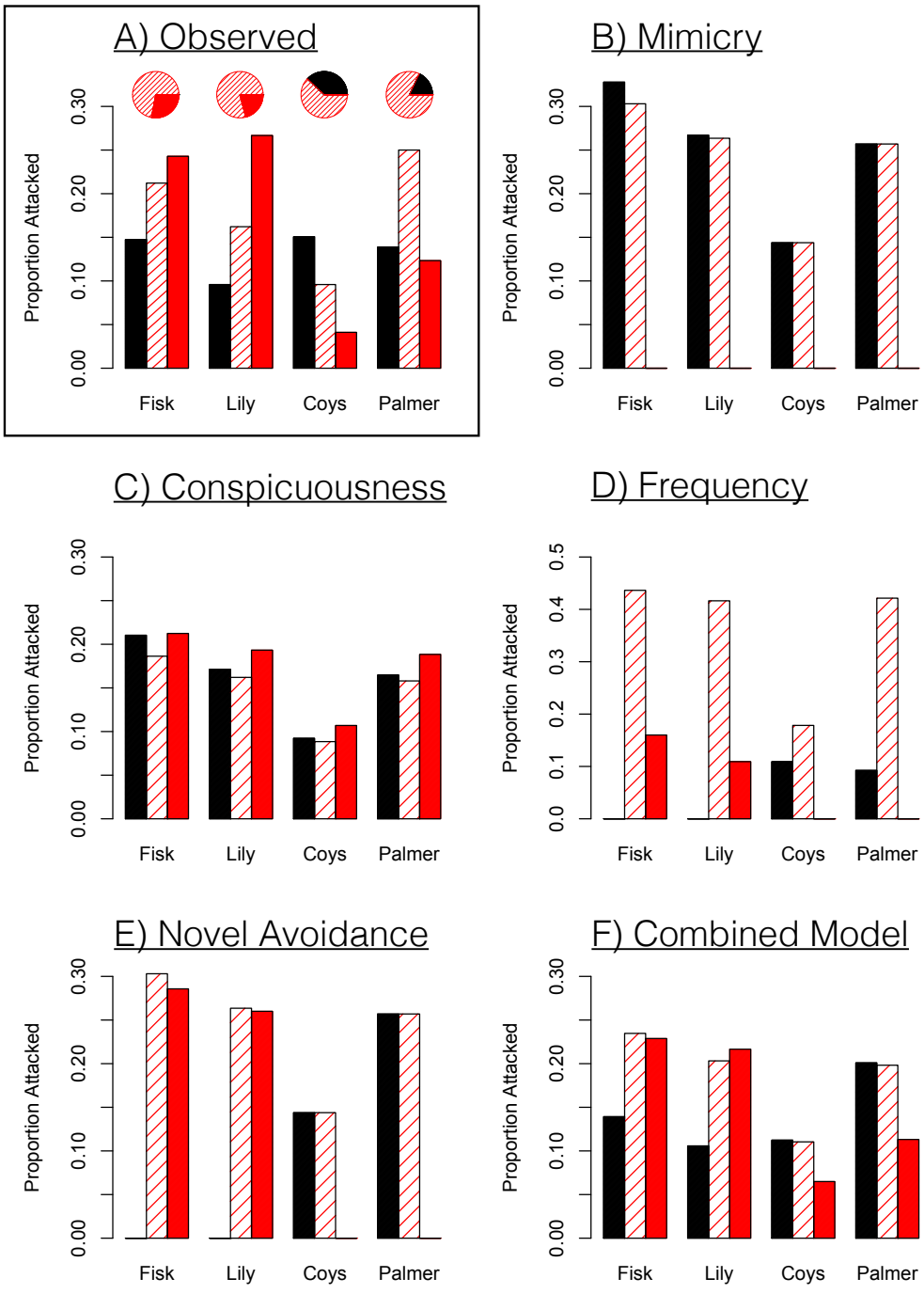

Figure 3. Predator attacks A) observed, B) predicted under the Mimicry Hypothesis, C) the Conspicuousness Hypothesis, D) the Frequency Hypothesis, E) the Novel Avoidance Hypothesis, and F) the Combined Hypothesis. Black bars represent the unstriped color morph, striped bars represent the striped morph, and red bars represent the erythristic morph. The pie charts in panel 'A' denote the proportion of each *Plethodon cinereus* color morph locally present at each locality.

meters along 75 m transects. All replicas were placed five meters perpendicular to the transect line. Each locality had 15 transects and all transects had at least 20 m between them. After four (for Fisk Meadows) or five days (for Lily Pond, Coys Hill, and Palmer) replicas were retrieved, though not all replicas could be recovered. This may be due in part to predators removing replicas from the transects, but may also be in part a result of our inability to relocate replicas placed in heterogeneous terrain (e.g. among boulders or vegetation). In such cases where models could not be recovered, they were removed from the analysis. We then scored replicas as either attacked if mammalian incisors or other indications of teeth could be identified in the clay, or not attacked if mammalian marks were absent (for similar scoring procedures, see: Brodie, 1993; Pfennig *et al*., 2001; Kutcha, 2005).

Expected Attacks for Each Hypothesis

Figure 1 provides a general schematic of attack frequencies under each predation hypothesis. To quantitatively characterize these into the expected attack rates under each hypothesis for our dataset, the following procedure was utilized. The Mimicry Hypothesis states that predators will avoid attacking prey that resemble unpalatable models (Bates, 1862). Thus, the null expectation for the Mimicry Hypothesis is predators will avoid mimics and attack non-mimic morphs at equal frequency. We consequently set expected attack rates at: 50% of observed attacks on unstriped replicas, 50% on striped replicas, and 0% on erythristic replicas (Figure 3B). The Conspicuousness Hypothesis states that attacks will be distributed among prey such that the most conspicuous prey will be attacked at the greatest frequency (Endler, 1978). We used previously published estimates of salamander

conspicuousness against local backgrounds from the perspective of mammal predators (Kraemer and Adams, 2014) to estimate relative conspicuousness. Erythristic *P. cinereus* are most conspicuous (conspicuousness score = 46.1), unstriped *P. cinereus* are moderately conspicuous (conspicuousness score = 43.8), and striped *P. cinereus* are least conspicuous (conspicuousness score = 41.0; see Kraemer and Adams, 2014). From these estimates of relative conspicuousness we set expected attack rates at: 35% on erythristic replicas (Figure 3C), 33% of attacks on unstriped replicas, and 31% on striped replicas. The Frequency Hypothesis states that most attacks will occur on morphs that are most common in the environment, such that common morphs will be attacked frequently, rare morphs will be attacked rarely, and novel morphs will be avoided (Allen, 1988). We thus set separate expected attack rates for each morph by locality (Figure 3D), with expected attack rates corresponding to the relative frequency of each morph naturally found at that locality as determined previously (Kraemer *et al.* in preparation; Figure 3A). The Novel Avoidance Hypothesis states that predators will avoid morphs not previously encountered (Marples *et al*., 2007). We classified morphs naturally present at a locality as 'previously encountered' by predators and thus predicted that they would not be avoided. Conversely, we considered novel morphs at a locality as 'not previously encountered' and predicted that they would be avoided by predators. For example, at the Fisk locality unstriped *P. cinereus* are absent, while striped and erythristic salamanders are present. We thus set attack rates at Fisk as 0% of observed attacks on unstriped replicas, 50% attacks on striped replicas, and 50% attacks on erythristic replicas (Figure 3E). The Null Hypothesis states that coloration and pattern will not influence attacks on potential prey (Endler, 1986). This hypothesis is derived from cases where predators locate prey items primarily using other senses (such as smell), or if visual

cues not related to coloration are utilized. As such, the Null Hypothesis predicts equal attacks across color morphs, or 33% of the attacks on unstriped replicas, 33% on striped replicas, and 33% on erythristic replicas. Finally, the Combined Hypothesis states that several factors jointly influence predator attacks on different color morphs, and thus predicts attack rates intermediate between the above predictions (Figure 3F; see *Combined Hypothesis* below).

Statistical Analyses

We evaluated observed predation rates on salamander replicas relative to alternative hypotheses using several analytical approaches. First, we determined which hypotheses provided the best 'fit' to the observed data using AIC scores. Next, we performed a series of pair-wise likelihood ratio tests (LRTs). These tests compared the likelihood of the observed data given each model of predator behavior through a non-nested LRT approach (*see Simulation LRT*). Finally, because not all biological hypotheses were mutually exclusive, there existed the possibility that some combination of these hypotheses provided the best explanation of the observed data. We therefore developed a novel likelihood procedure to combine non-exclusive biological hypotheses (see *Combined Hypothesis* below).

Simulation LRT

Simulation LRT is an approach where non-nested models may be compared using likelihood ratio tests (Williams, 1970; Lewis *et al*., 2010). The procedure consists of six steps, which are outlined briefly here. First, for two non-nested models, 'A' and 'B,'1) calculate the likelihood of models 'A' and 'B' given the parameters of the models and observed data. Next, simulate a large number of datasets under the null model 'A,' fit each

dataset to the null ‘A’ and alternative ‘B’ models, and calculate Likelihood Ratio Test Statistics (LRTS) for each simulated dataset as well as the observed dataset. The proportion of LRTS from the simulated datasets that are more extreme than the observed data is then estimated, and if the observed LRTS is more extreme than 95% of the simulated datasets, the LRT is judged as ‘significant.’ Finally, the steps are repeated with the role of ‘null’ and ‘alternative’ model reversed.

The procedure above provides a means of evaluating the fit of data to multiple models that are not statistically nested (as is the case here). As noted by Lewis *et al*. (2010), this analysis has four potential outcomes. 1) The LRT with A as the null model is non-significant, but the LRT with B as the null is significant. In this case, model A is a better fit than model B. 2) The LRT with B as the null model is non-significant, but the LRT with A as the null is significant. In this case, model B is a better fit than model A. 3) If both LRTs are significant, neither model fits the data well. 4) If neither LRT is significant, the two models cannot be distinguished given the available data. We used this procedure on each pair of the hypotheses described above to determine which hypothesis (if any) provided a better explanation for the observed attack rates.

Combined Hypothesis

The LRT procedure described above is quite flexible, in that it allows one to compare the fit of non-nested models to data using likelihood ratio tests. However, the method assumes that all models are mutually exclusive, which is not always case. With respect to predation, predators may use prey coloration differently throughout the predation process, which suggests the above hypotheses (Conspicuousness, Frequency, Mimicry, Novel

Avoidance) are not necessarily exclusive of one another (Endler, 1986). Because of this, predators may, in essence, combine information from multiple sources, effectively utilizing multiple strategies that fall within the previously defined hypotheses. In such cases, the best explanation for the observed attack rates would be from a model that does not exclusively describe one or another scenario, but rather combines multiple models (akin to model averaging using AIC weights). To address this possibility, we developed a likelihood procedure that identified the best fitting model (based on likelihood) where this model consisted of combinations of the previously stated hypotheses. Procedurally, this was accomplished by incorporating weights (w) for each model, which were multiplied by the parameters of each hypothesis (i.e. Conspicuousness: β_{conspic}, Frequency: β_{freq}, Mimicry: β_{mim}, Novel Avoidance: β_{novel}). These weights were then adjusted to maximize the likelihood of a combined hypothesis, with the constraint that the weights sum to 1.0. Thus, the best fitting combined model was found as:

$$\beta_{combined} = w_{\text{conspic}}\beta_{\text{conspic}} + w_{\text{freq}}\beta_{\text{freq}} + w_{\text{mim}}\beta_{\text{mim}} + w_{\text{novel}}\beta_{\text{novel}},$$

with $\beta_{combined}$ signifying the parameters of a combined model that maximizes the likelihood of a hypothesis incorporating aspects of each single hypothesis. Note that for a 'pure' model, the weight for that component would be w = 1.0, and the weights for the remaining model contributions would be w = 0.0. We implemented this procedure using the 'optim' function in the 'stats' package found in 'R.' We then compared the fit of the combined hypothesis to each 'pure' hypothesis using likelihood, Akaike Information Criterion (AIC), and simulation LRT. All analyses were conducted in R 3.0.2 (R Development Core Team, 2013).

Results

Likelihood and AIC

We found likelihood scores and AIC scores reflected similar patterns of fit among single models (Table 1). In both cases, the Novel Avoidance Hypothesis was a much better fit to the observed data than were the remaining models. The next best-fitting models (Null and Conspicuousness) were >6 ΔAIC units from the Novel Avoidance Hypothesis, implying that the Novel Avoidance Hypothesis represented a substantially better fit to the observed attack rates (Table 1). Further, two models that described strict avoidance of one morph (Mimicry and Frequency Hypotheses) provided the poorest fit to the data, implying that these models did not describe how predators attacked potential prey in this system (Table 1).

Table 1. Likelihood and AIC scores for each hypothesis. Both approaches indicate that, of the hypotheses tested, Novel Avoidance is the best fitting single hypothesis, while the Combined Hypothesis best fits the observed data over all single hypotheses.

Model	**-lnL**	**AIC**
Combined	-3.64	15.28
Novel Avoidance	-7.65	19.31
Null	-10.58	25.16
Conspicuousness	-10.72	25.44
Mimicry	-19.18	42.36
Frequency	-35.14	74.28

Simulation LRT

Pairwise comparisons of models through simulation LRT corroborated the findings described above, indicating that the Mimicry and Frequency hypotheses were particularly poor predictors of the observed data (Table 2). In no comparisons did either of these models fit better than the alternative. Comparisons between the remaining single models were more equivocal, and we were unable to determine whether the Novel Avoidance, Conspicuousness, or Null models provided the best fit.

Table 2. Pairwise comparisons of each hypothesis using simulation-based LRT. 'Conspic.' refers to the Conspicuousness Hypothesis and 'Novel Avoid.' refers to the Novel Avoidance Hypothesis. Row names that correspond to each cell indicate the model treated as the 'null' hypothesis, while column names denote the 'alternative' hypothesis. Note that the Combined Hypothesis is the best fit among all comparisons, because all comparisons with the Combined Hypothesis as the null are non-significant and all comparisons with the Combined Hypothesis as the alternative are significant.

	Frequency	Mimicry	Conspic.	Novel Avoid.	Null	Combined
Frequency	-	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Mimicry	**<0.001**	-	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Conspic.	0.122	0.478	-	**0.007**	0.506	**<0.001**
Novel Avoid.	0.798	**<0.001**	**<0.001**	-	**<0.001**	**<0.001**
Null	0.343	0.877	0.682	**0.020**	-	**<0.001**
Combined	0.774	0.607	0.916	0.622	0.741	-

Combined Hypothesis

Interestingly, the combined hypothesis that best predicted our observed data was most strongly influenced by the Conspicuousness Hypothesis ($w_{conspic} = 0.61$) and Novel Avoidance Hypothesis ($w_{novel} = 0.39$), with no contribution from the remaining hypotheses. Consequently, only the parameters from the Conspicuousness and Novel Avoidance hypotheses were considered in calculating the AIC score for the combined hypothesis. Note that the best-fitting combined hypothesis does not weight the Conspicuousness and Novel Avoidance hypotheses according to AIC or likelihood scores, but by the predictions made by each resulting model. Both likelihood and AIC scores indicated that the Combined Hypothesis was a far better predictor of our observed data than any single hypothesis. Likewise, the Combined Hypothesis was a better fit than any single hypothesis as indicated by simulation LRT.

Overall, results from AIC, simulation LRT, and combined LRT consistently group the Novel Avoidance Hypothesis among the best-fitting hypotheses. Results from the combined LRT test found the highest support for the combination of the Novel Avoidance and Conspicuousness hypotheses.

Discussion

Selection on coloration and patterning can be strongly driven by predators (Endler, 1988), yet how multiple predators influence selection on the same color patterns is less well known (Endler, 1986). Importantly, prey color patterns that signify toxicity to some predator species may not carry the same indication of unpalatability to other predators (Pekár *et al*., 2011). In this study, we tested several hypotheses of selection on color pattern between

mammal predators and the salamander *Plethodon cinereus*. We found consistent support for the single hypothesis that mammals avoid novel and unfamiliar prey as has been observed in experimental settings (Chitty and Kempson, 1949; Mitchell, 1976), and we found the highest support for a combined hypothesis in which mammalian predators preferentially attack the most conspicuous prey while avoiding unfamiliar color morphs.

Avian predators avoid erythristic *P. cinereus* because they appear similar in coloration to the toxic salamander *Notophthalmus viridescens* (Brodie and Brodie, 1980; Tilley *et al*., 1982). Selection for Batesian mimicry in *P. cinereus* may or may not extend to additional predator species, such as mammals. We tested this hypothesis by fitting our observed data to a model in which mammals avoided erythristic replicas while not discriminating between either striped or unstriped replicas. We found no support for this hypothesis alone or as a component of the Combined Hypothesis. The lack of support for mimicry between erythristic *P. cinereus* and *N. viridescens* from the perspective of mammalian predators suggests that mammals do not associate erythristic *P. cinereus* with unpalatability, which aligns well with recent research in the system. Kraemer and Adams (2014) found evidence that the dichromatic mammalian visual system allows mammals to differentiate salamander species from each other and common backgrounds on the basis of brightness, but not coloration. This is significant with regards to mimicry because erythristic *P. cinereus* and *N. viridescens* are similar with respect to color, while they differ substantially with respect to brightness (Kraemer and Adams, 2014). Predators that select for mimicry between *P. cinereus* and *N. viridescens* must be capable of identifying prey by color while foraging, which dichromatic mammals appear unable to do. When paired with our

findings, this research strongly indicates that dichromatic mammals do not select for mimicry in this system.

By contrast, in this study we found strong statistical support for a composite hypothesis that incorporates aspects of two modes of prey selection, Novel Avoidance (Marples *et al*., 2007) and Conspicuousness (Endler, 1978), with Novel Avoidance previously observed in mammal taxa (Chitty and Kempson, 1949; Barnett, 1958). These results are intriguing because mammals are well known to use olfactory cues while foraging (Pyare and Longland, 2001; Hughes *et al*., 2010). However, our data support two hypotheses that indicate mammals also utilize visual cues, specifically by attacking prey that most strongly contrast with local backgrounds and that are most familiar in appearance. This result implies that mammals use visual cues both during the detection and identification phase of foraging, which allows for the possibility that these predators use visual cues of their prey at different stages of single predation events (Endler, 1986). For example, a color pattern that influences a predator's ability to first detect potential prey (the Conspicuousness Hypothesis; Endler, 1978), may also impact whether that predator then recognizes the potential prey as a prey item (i.e. the Novel Avoidance, Batesian Mimicry, or Frequency hypotheses; summarized in Endler, 1986). Our findings suggest selection on *P. cinereus* by mammalian predators is complex, with a combination of directional selection favoring inconspicuous individuals and frequency-dependent selection favoring novel color morphs. Interestingly, this selection could result from a complex predator community consisting of several species with each species responding to salamander visual cues differently, a predator community with behavioral plasticity among individuals, or a single predator type that incorporates visual cues relating to prey conspicuousness and novelty at every encounter with potential

prey. The next, and perhaps most difficult, research direction will be to identify all members of these salamander predator communities and characterize how each interacts with individual salamander prey.

Salamander conspicuousness and novelty to predators are strongly influenced by local community structure and abiotic factors, which may result in selection pressures that differ among localities. For example, conspicuousness is determined by the contrast between prey and background (Endler, 1978). Importantly, background may differ by locality and therefore be influenced by factors such as the composition of tree species, precipitation, vegetative cover, and time of year (Endler, 1993). Likewise, the contribution of novel avoidance in the best-fitting combined hypothesis supports the hypothesis that predators avoid prey they have not previously encountered (e.g. Mitchell, 1976; Lindström *et al*., 2001). Furthermore, when classified as either local (if corresponding *P. cinereus* morphs were present at the locality) or novel (if corresponding *P. cinereus* morphs were absent), local salamander replicas were twice as likely to be attacked than novel replicas (local proportion attacked = 0.23; novel proportion attacked = 0.11). Thus, our Combined Hypothesis indicates that mammal predators are strongly influenced by local factors. Variation in potential backgrounds and prior predator experience may contribute to the variability observed between natural predator-prey studies conducted under similar, but not identical conditions (e.g., as seen between Saporito *et al.,* 2007 and Hegna *et al*., 2013).

Plethodon cinereus are likely hunted by several predator taxa (Lotter and Scott, 1977), with each species potentially directing the evolution of *P. cinereus* phenotype along a different evolutionary trajectory. Previous research suggests that bird predators select *P. cinereus* coloration such that erythristic individuals evolve coloration that is similar to *N.*

viridescens (Brodie and Brodie, 1980; Tilley *et al.,* 1982; Kraemer and Adams, 2014). Here, we present evidence that mammal predators may select for inconspicuousness in *P. cinereus* as well as for novel color morphs. This complex selective regime may contribute to the maintenance of polymorphism in *P. cinereus* through variation in predator communities within and among localities as well as consistent selection for novel, unfamiliar color morphs.

Color polymorphism is widespread in many species in addition to *P. cinereus* (e.g. moths: Poulton, 1890; land snails: Cain and Sheppard, 1954; desert reptiles: Norris and Lowe, 1964), though the mechanisms maintaining polymorphisms are hotly debated (Bond, 2007). Polymorphisms can be maintained by frequency-dependent selection (such as novel avoidance; Marples *et al*., 2007), variation in prey community structure (Merilaita and Kaitala, 2002), multiple predators with variable behavior (Nokelainen, 2014), gene flow among populations (Slatkin, 1987), and habitat heterogeneity (Sandoval and Nosil, 2005). In many species, including *P. cinereus*, polymorphism is likely maintained by a complex bricolage of the above mechanisms. Future studies will have to account for the non-exclusivity of these mechanisms, as we have, in order to construct realistic theoretical models of polymorphism.

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CHAPTER 6

GENERAL CONCLUSIONS

Batesian mimicry is a complex phenomenon characterized by antagonistic interactions between mimics, models, and their predators. This complexity inspires evolutionary research through such questions as: how do predators view mimics, and what aspects of model phenotype do mimics resemble? Does mimicry influence the evolution of model phenotype? Can model unpalatability impact the evolution of mimicry? What impact do multiple predators have on the evolution of mimic phenotype? In this dissertation, I utilized several complimentary procedures and evaluated multiple hypotheses to explore the evolution of Batesian mimicry, and its consequences, in salamanders.

In Chapter 2, I examined how several potential predators perceive the resemblance between mimics and models using the model salamander *Notophthalmus viridescens* and putative Batesian mimic, the erythristic color morph of *Plethodon cinereus*. I tested several predictions of mimicry regarding predator perception of salamanders and their backgrounds. First, I found mimicry to be restricted to coloration, but not brightness, components of salamander phenotype. Second, only bird predators appeared able to discriminate between the colors of models and non-mimic *P. cinereus*. Third, estimates of salamander conspicuousness, or contrast to surrounding background, were background-dependent, corresponding to predictions only for backgrounds against which salamanders are most active. These results supported the hypothesis that birds influence the evolution of Batesian mimicry in *P. cinereus*, as they are the only group examined capable of differentiating *N. viridescens* and non-mimetic *P. cinereus*. Additionally, patterns of conspicuousness

suggested that selection from predators may drive the evolution of conspicuousness in this system. This study confirmed the expectation that the visual abilities of predators may influence the evolution of Batesian mimicry, but the role of conspicuousness may be more complex than previously thought.

Conspicuousness is a common feature of unpalatable species, and is thought to evolve to reduce confusion with palatable species (potential Batesian mimics). This hypothesis hinges on the 'cost of conspicuousness,' in which conspicuousness increases predation likelihood more in palatable species than unpalatable species. Under this scenario three patterns are expected: 1) a positive relationship between mimic and model conspicuousness, 2) greater model conspicuousness in the presence of mimics, and 3) a mismatch between model and mimic conspicuousness, with mimics appearing less conspicuous. In Chapter 3, I tested these predictions in the salamander mimicry system involving *Notophthalmus viridescens* (model) and *Plethodon cinereus* (mimic). All predictions were supported, indicating that selection for Batesian mimicry not only influences the evolution of mimics, but also the models they resemble. These findings suggest that mimicry may play a role in the evolution of model warning signals in this system.

Several additional factors can temper the evolution of Batesian mimicry, including model toxicity. In Chapter 4, I examined how model toxicity may influence the evolution of mimicry in *P. cinereus.* Model toxicity is thought to influence both the occurrence of mimicry and the evolution of mimetic phenotypes, and mimicry is most likely to persist when models are particularly toxic. Additionally, model toxicity may influence the evolution of mimetic phenotype in one of two ways. First, model toxicity may drive the evolution of mimetic fidelity such that mimicry will be most accurate when models are most toxic.

Alternatively, model toxicity may allow inaccurate mimicry to evolve through a mechanism termed 'relaxed selection.' I tested these hypotheses between the model *N. viridescens* and the mimic *P. cinereus*, in which *N. viridescens* toxicity takes the form of tetrodotoxin. Surprisingly, though I discovered spatial variation in model toxicity, I found no support for the hypotheses that model toxicity influences either the occurrence of mimicry or the evolution of mimic phenotype. Instead, variation in predator communities among localities or modern changes in environmental conditions may contribute to the patchy occurrence of mimicry in *P. cinereus*. Finally, limitations of predator perception or variation in the rate of phenotypic evolution of models and mimics may account for the evolution of imperfect mimicry in this salamander species.

In addition to model warning signals, model unpalatability, and mimic phenotype, variation in the predator community may influence mimicry evolution. In particular, predators can influence selection on prey coloration and patterning, yet how selection may differ among predators is seldom considered. Furthermore, prey color patterns that indicate unpalatability to some predator species may not carry the same signal for other predators. In Chapter 5, I tested several hypotheses of selection on coloration and pattern between mammal predators and *P. cinereus*. I fit each hypothesis against field observations of mammalian predation on salamander clay replicas. I then developed a novel analytical procedure that enabled the combination of multiple hypotheses in a likelihood framework. I found that mammals did not follow any individual hypothesis proposed, including the hypothesis of mimicry. Instead, mammals used visual cues while foraging by avoiding unfamiliar, novel prey and attacking conspicuous prey. I concluded that mammals may help to maintain color polymorphism within populations of *P. cinereus* by avoiding novel,

unfamiliar color morphs. Additionally, I inferred that selective pressures from multiple predators and variation in predator communities among sites may contribute to the maintenance of color polymorphism within and among localities in *P. cinereus*.

Through this research, I found strong support for the hypothesis of Batesian mimicry between the erythristic color morph of *Plethodon cinereus* and *Notophthalmus viridescens* efts. Further, I discovered evidence that model and mimic phenotypes co-evolve, with mimics evolving warning signals similar to models, while models evolve warning signals that distinguish them from mimics. Contrary to theoretical predictions, I found no link between model toxicity and mimicry, which demonstrates the importance of testing biological theory in natural systems. Finally, I found that alternative predators must be considered, as they may direct the evolution of mimicry even, and perhaps especially, when they influence selection on mimic phenotype in ways not predicted by mimicry. In light of these findings, many questions remain. Why do *P. cinereus* mimic *N. viridescens* coloration, but not brightness? How likely is the evolution of mimicry? What determines the components of model warning signals that mimics counterfeit? Why are mimics not always found at the same sites as models? Cases of mimicry are exceptional examples of the power of natural selection, and as Henry Walter Bates referred to them, 'these apparently miraculous, but always beautiful and wonderful, mimetic resemblances' will continue to yield insight into the evolutionary process.